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LABORATORY SCREENING OF LOCAL STREPTOMYCES ISOLATES FOR ANTIBIOTIC ACTIVITY AGAINST XANTHOMONAS ORYZÆ (UYEDA AND ISHIYAMA) DOWSON AND PYRICULARIA ORYZÆ CAV.

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TWO PLATES AND TWO TEXT FIGURES

ABSTRACT

Thirty-six Streptomyces isolates showed varied degrees of antibiotic activity against *Xanthomonas oryzæ*, the causal organism of rice leaf blight. In the test on *Pyricularia oryzæ* or the organism causing rice blast only 39 isolates showed antibiotic activity. Against both pathogens, 85 isolates were found active in varying degrees. Twenty-nine isolates were found promising antibiotic producers. Medium I was found best for production of antibiotic substances against *X. oryzæ*.

INTRODUCTION

Antibiotics have shown great prospects in the control of rice diseases, principally bacterial leaf blight caused by *Xanthomonas oryzæ* (Uyeda and Ishiyama) Dowson and blast caused by *Pyricularia oryzæ* Cav. In Japan, for example, blasticidin S

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place of maltose or glucose. All the media were adjusted to pH 7.0 to 7.2 before sterilization at 121°C for 15 min.

TABLE 1.—*Fermentation media used in shake-flask screening.*

Ingredients	Medium *							
	I	II	III	IV	V	VI	VII	VIII
Sucrose	1	1	.....	1.5	.....	1.5	.....	1
Glycerol	.....	.....	1	.....	.....	1	4	.....
Maltose	.....	.....	.....	.....	1	.....	.....	.....
Starch	1	1	.....	.....	.....	.....	.....	1
Soybean	1.5	.....	0.5	1.5	1	1.5	2.5	1.5
Corn steep liquor	.....	.....	0.5	.....	.....	.....	.....	.....
Peptone	.....	0.75	.....	.....	.....	.....	0.5	.....
Beef extract	.....	0.75	.....	.....	.....	.....	.....	.....
Dried yeast	.....	.....	.....	.....	0.25	0.5	.....	.....
$(NH_4)_2SO_4$	.....	.....	.....	.....	0.5	0.5	.....	.....
NaCl	0.3	0.3	0.5	0.3	.....	0.5	0.5	0.3
$CaCO_3$	.....	.....	0.2	.....	1.4	0.4	.....	.....
$FeHPO_4$	0.1	.....	0.2	0.1	0.03	.....	.....	0.01
$MgSO_4$	0.05	.....	.....	0.05	.....	.....	.....	0.05
KCl	.....	.....	.....	.....	0.4	.....	.....	.....

* Amount of ingredient is expressed in percentage.

The flasks were incubated on the reciprocating shaker and the culture brew sampled on the 3rd, 5th, and 7th days. The samples were tested for antibiotic activity by the paper-disc assay as used by Pridham *et al* (1956). Sterile 6.5-ml paper discs were dipped into the culture brew and were placed equidistant with each other on the agar surface of the assay plates. Incubation of plates and determination of antibiotic activity were done in the same way as in the primary screening.

RESULTS AND DISCUSSION

Primary screening. The primary screening was conducted to (*a*) find antagonistic Streptomyces isolates, (*b*) classify the degree of antibiotic activity, and (*c*) select the promising isolates for the next screening.

A total of 272 Streptomyces cultures was tested for their antibiotic activities in the solid medium against the two plant pathogens. One hundred sixty isolates or 58.8 per cent were found showing inhibitory action of varying degrees against either *X. oryzæ* or *P. oryzæ* or both. Regardless of the test organisms used, this percentage of antagonistic Streptomyces is higher than what Santos and de Leon (1962) and Emerson *et al* (1946) obtained. No conclusion, however, can be drawn

about the abundance of the anti-Xanthomonas and anti-Pyricularia Streptomyces in Philippine soils because of the limited number of isolates tested.

The antibiotic activities of the 160 active isolates were classified according to size of inhibition zones produced (Table 2). "Very Strong" activity was assigned for zones of inhibition ranging from 41 mm and above, "Strong" activity for 30 to 40 mm, "Moderate" activity for 19 to 29 mm, and "weak" activity for 18 mm and below. It was found that of the 36 isolates that exhibited activity only against *X. oryzæ*, 10 gave Strong activity, 16 had Moderate activity, and 10 had Weak activity. Not one of the isolates gave Very Strong activity. On the other hand, of the 39 isolates found active only against *P. oryzæ*, 2 had Very Strong activity, 13 had Strong activity, 17 had Moderate activity, and 7 had Weak activity.

It will be noted that some isolates exhibited antagonistic action on both *P. oryzæ* and *X. oryzæ*, a fungus and a bacterium, respectively. This kind of action, according to Emerson *et al* (1946), may be attributed to the production of a single antibiotic or the production of more than one. Table 2 shows that of the 85 isolates found possessing such property, 2 had

TABLE 2.—*Distribution of activity of 160 Streptomyces isolates.*

Activity class[1]	Number of isolates active against			Total
	X. oryzæ	*P. oryzæ*	Both[2] pathogens	
Very strong	0	2	2	4
Strong	10	13	25	48
Moderate	16	17	47	80
Weak	10	7	11	28
Total	36	39	85	160

[1] Activity was classified according to size of inhibition zone—very strong, 41 mm and above: Strong 30–40 mm; Moderate, 19–20 mm: and Weak, 18 mm and below.

[2] Data based on the highest zone of inhibition produced by the isolates against *P. oryzæ* or *X. oryzæ*.

Very Strong activity, 25 had Strong activity, 47 had Moderate activity, and 11 had Weak activity. Since most antibiotics so far reported are effective against either *P. oryzæ* only or *X. oryzæ* only, the investigation on the possibility of producing antibiotics active against both pathogens would be of much interest.

From the 160 active isolates, selection of the promising isolates for the secondary screening was made. A total of 52 isolates, 4 of which have Very Strong activity and 48 which have Strong activity (Table 2), was chosen. Their sources and antibiotic activities in the solid medium against the two plant pathogens are shown in Table 3.

Plate 1, figs. 1 and 2, shows typical growth inhibition of *X. oryzæ* and *P. oryzæ* by the Streptomyces isolates grown in solid medium as tested by agar-plug assay.

Secondary screening.—The secondary screening was employed to (*a*) determine which of the isolates could show antibiotic activity in shaken liquid media, (*b*) classify the degree of antibiotic activity, (*c*) select the promising isolates for further studies, and (*d*) obtain information concerning media requirements for antibiotic production.

The results of the experiment show that all except 5 of the 52 isolates produced culture liquors that exhibited activity of varying degrees against the two pathogens. The failure of the 5 isolates to produce active culture liquors may be due to unfavorable media, pH, temperature, etc. This phenomenon can be observed in the work of Emerson *et al* (1946) when antagonistic actinomycetes and molds were grown in liquid culture media.

The antibiotic activities of the isolates were classified in the same manner as in the primary screening. Out of the 47 isolates that produced active culture liquors, 29 exhibited Strong and Very Strong activity in at least one of the media against at least one of the test organism as shown in Table 4. All these isolates were considered for further studies. The data on the remaining 18 isolates which produced Weak to Moderate activity in at least one of the media against at least one of the test organisms were not presented because too much space would have been required.

Of the 8 liquid media used, it is apparent from the results that Medium I is the best for production of antibiotic substances by the isolates against *X. oryzæ* in that highest percentage of total active isolates as well as highest percentage of isolates that gave Very Strong and Strong activity was obtained (Fig. 1). On the other hand, none of the media proved superior to the others for production of antibiotic substances against *P. oryzæ* as shown in Fig. 2. Though Medium II has the lowest total percentage of active isolates, it seems to be

TABLE 3.—*Antibiotic activity of 52 Streptomyces isolates tested in solid medium against two test organisms.*

Source	Isolate number	Test organism	
		X. oryzæ	P. oryzæ
Sexmoan, Pampanga	S-70-39	++++	+++
Ditto	70-26,-25,-27, -30,-32,-38,-40	+++	+++
Ditto			
Pasay City	60-62		
Parañaque, Rizal	61-50		
Gapan, Nueva Ecija	69-64		
Tarlac, Tarlac	70-21,-23	++	+++
Sexmoan, Pampanga	70-28,-29,-31		
San Antonio, Zambales	70-41		
Papaya, Nueva Ecija	69-101	++++	+
Lian, Batangas	70-115,-132		
Imus, Cavite	69-46		
Pasig, Rizal	69-112,-114,-117	+++	+
San Juan, Rizal	69-131		
Molino Farms, Cavite	70-6		
Naujan, Mindoro	62-20		
Silang, Cavite	69-13,-15		
Manila	68-53		
Gapan, Nueva Ecija	69-51,-58	+++	—
Cabanatuan, Nueva Ecija	69-70		
Papaya, Nueva Ecija	69-75		
Lian, Batangas	70-121		
Marikina, Rizal	62-84	+	+++
Lian, Batangas	70-134,-135	—	++++
Zamboanga	61-1,-8		
Puerto Galera, Palawan	62-1		
Baco, Mindoro	62-4		
Naujan, Mindoro	62-22,-23	—	+++
Calapan, Mindoro	62-27		
Cabanatuan, Nueva Ecija	69-72		
San Jose, Nueva Ecija	69-88,-93		
Kawit, Cavite	69-106		
Lian, Batangas	70-129,-130,-136		

LEGEND: ++++ (Very Strong), inhibition of 41 mm and above; +++ (Strong), inhibition of 30-40 mm; ++ (Moderate), inhibition of 19-29 mm; + (Weak), inhibition of 18 mm and below; — (Inactive), no inhibition.

suitable for antibiotic production in that it has the highest percentage of isolates with Very Strong activity. Other media such as Media III, IV, V, VII, and VIII failed to favor production of antibiotic substances with Very Strong activity against the pathogen. For any particular isolate, however, there seemed to be a medium really suitable for antibiotic production.

Typical growth inhibition of *X. oryzæ* and *P. oryzæ* by active culture brew of the isolates as tested by the paper-disc assay is shown in Plate 2, figs. 1 and 2.

TABLE 4.—*Antibiotic activity[1] of culture liquors of 29 Streptomyces isolates in eight fermentation media against two test organisms.*

Isolate number	Test organism[2]: *X. oryzæ*								Test organism[2]: *X. oryzæ*							
	I	II	III	IV	V	VI	VII	VIII	I	II	III	IV	V	VI	VII	VIII
9-70-39	++++	++++	++	+	+	+	+	+	+	—	+	+	+	+	+	+
70-25	—	++	++	++	+	+	—	+	+	+	+	+	+	+	+	+
70-26	++++	++++	++	+	—	+	—	+	+	+	+	+	+	+	+	+
70-27	+++	++++	+++	++	++	++	+	+	+	+	+	+	+	+	+	+
70-30	+++	+++	++	+++	+	+	+	+	+	+	+	+	+	+	+	+
70-32	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+
70-38	++	++	+	+	—	+	—	+	+	+	+	+	+	+	+	+
60-62	—	+	+	—	—	+	+	+	+	+	+	+	+	+	+	+
70-21	+++	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+
70-23	++++	++++	++++	+++	+	+	+	+	+	+	+	+	+	+	+	+
70-28	+++	+++	+++	++	—	+	+	+	+	+	+	+	+	+	+	+
70-29	+++	+++	+++	+++	—	+	+	+	+	+	+	+	+	+	+	+
70-31	++++	++++	+++	++++	+	+	+	+	+	+	+	+	+	+	+	+
70-115	++	+	+	+++	++	+	—	+	+	+	+	+	+	+	+	+
62-84	++	—	—	—	—	—	—	+	—	—	+	+	+	+	+	+
70-122	+++	—	++	+++	++	—	—	—	—	—	+	+	+	+	+	+
59-117	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+	+
62-20	++++	++	—	—	—	—	+	—	—	—	—	+	+	+	+	+
70-121	+++	—	++	+	++	—	—	+	—	—	—	—	—	—	—	+
61-1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
61-8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
62-1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
62-4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
62-22	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
62-23	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
62-27	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
69-28	+++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
69-33	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
69-106	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

[1] Classified according to size of inhibition zone: ++++ (Very Strong), 41 mm and above; +++ (Strong), 30–40 mm; ++ (Moderate) 19–29 mm; + (Weak), 18 mm and below; — (Inactive), no inhibition.

[2] Data represent the highest inhibition zone obtained from three samples tested.

Note: The 23 isolates which gave negative to moderate activity against the two test organisms in eight media were not presented in the table.

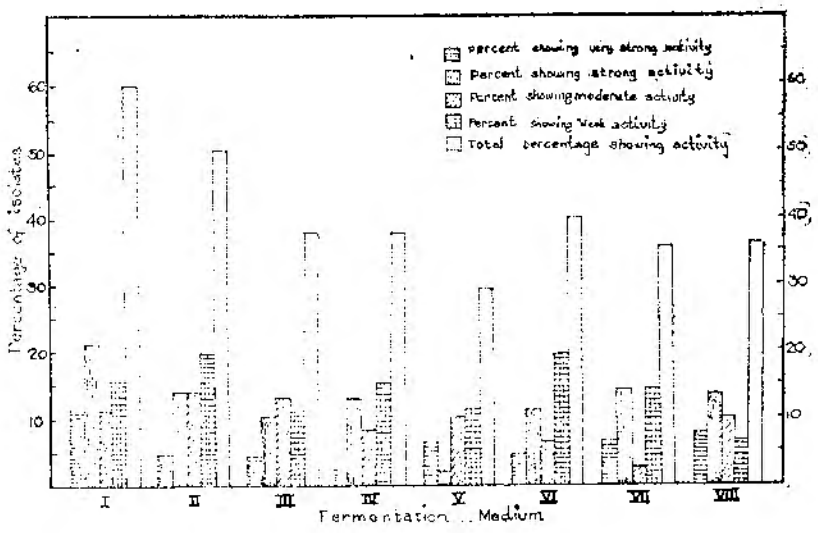

FIG. 1. The degree of antibiotic activity of the Streptomyces isolate in eight fermentation media against *X. oryzae*.

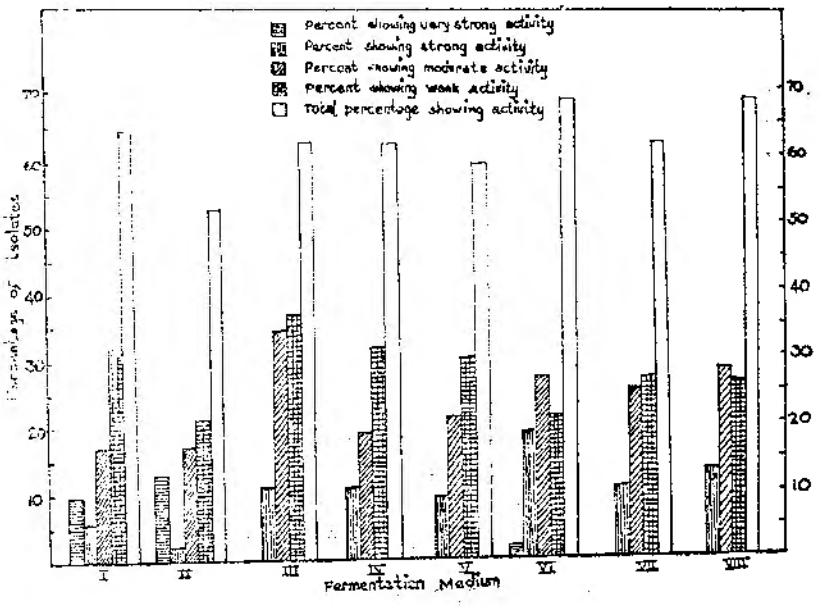

FIG. 2. The degree of antibiotic activity of the Streptomyces isolates in eight fermentation media against *P. oryzae*.

SUMMARY

Out of 272 Streptomyces isolates tested for antibiotic activity in the solid medium against the two rice plant pathogens, 36 were found active in varying degrees against *X. oryzæ* only, 39 against *P. oryzæ* only, and 85 against both pathogens.

Of the 52 isolates tested under the shake-flask screening using 8 liquid media, 29 were found to be promising antibiotic producers and were considered for further studies.

Of the 8 liquid media used, Medium I is the best for production of antibiotic substances by the isolates against *X. oryzæ*. No medium proved superior to the others for production of antibiotic substances against *P. oryzæ*.

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REFERENCES

AIZAWA, S., Y. NAKAMURA, S. SHIRATO, R. TAGUCHI, I. YAMAGUCHI, and T. MISATO (1969). Aabomycin, a new antibiotic. I. Production, isolation and properties of aabomycin A. Jour. Antibiot., Ser. A 22 (10): 457–462.

EAMICHIT, S. (1969). A study on antibiotic tolerance in some isolates of Xanthomonas oryzæ (Uyeda et Ishiyama) Dowson and pathogenicity test of some antibiotic-resistant strains. Short-term research paper, Dept. of Plant Pathology, IRRI. Unpublished.

EMERSON, R. L., A. J. WHIFFEN, N. BOHONOS, and C. DEBOER (1946). Studies on the production of antibiotics by actinomycetes and molds. Jour. Bact. 52: 357–366.

HARA, T., T. NIIDA, K. SATO, S. KONDO, T. NOGUCHI, and K. KOHMOTO (1964). A new antibiotic, cranomycin. Jour. Antibiot., Ser. A. 17 (16): 266.

HASHIMOTO, T., M. KITO, T. TAKEUCHI, M. HAMADA, K. MAEDA, Y. OKAMI, and H. UMEZAWA (1968). Ablastmycin, a new anti piricularia antibiotic. Jour. Antibiot., Ser. A 21 (1): 37 43.

MISATO, T. (1969). The development of agricultural antibiotics in Japan. Japan Pesticide Information 1: 15–18.

PRIDHAM, T. G., L. A. LINDENFELSER. O. L. SHOTWELL., F. H. STODOLA, R. G. BENEDICT, C. FOLEY, R. W. JACKSON, W. J. ZAUMEYER, W. H. PRESTON, JR., and J. W. MITCHELL (1956). Antibiotics against plant disease. I. Laboratory and greenhouse survey. Phytopathology 46: 568 575.

SAKAGAMI, Y., H. SEKINE, S. YAMABAYASHI, Y. KITAURA, and A. UEDA (1966). The studies on bramycin. a new antifungal antibiotic. I. Isolation, purification, and properties of bramycin. Jour. Antibiot., Ser. A 19 (3): 99-103.

SAKAGAMI, Y., A. UEDA, S. YAMABAYASHI (1967). Studies on ikutamycin, a new antifungal antibiotic. I. Isolation and properties of ikutamycin Jour. Antibiot., Ser. A 20 (6): 299-303.

SANTOS, P. S., and W. DE LEON (1962). A survey of Philippine soils for antiobiotic-producing Streptomyces. Philip. Jour. Sci. 19: 241–253.

SHOJI, J., R. SAKAZAKI, M. MAYAMA, Y. KAWAMURA, Y. YASUDA (1970). A new antibiotic, bulgerin, active against phytopathogenic fungi. Jour. Antibiot., Ser. A 23 (6): 295 299.

SUZUKI, S., K. ISONO, J. NAGATSU, T. MIZUTANI, Y. KAWASHIMA, and T. MIZUNO (1965). A new antibiotic, polyoxin A. Jour. Antibiot., Ser. A 18 (3): 131.

TAKEUCHI, S., K. HIRAYAMA, K. UEDA, H. SAKAI, and H. YONEHARA (1958). Blasticidin S, a new antibiotic. Jour. Antibiot., Ser. A 11 (1): 1 5.

UMEZAWA, H., Y. OKAMI, T. HASHIMOTO, Y. SUHARA, M. HAMADA, and T. TAKEUCHI (1965). A new antibiotic, kasugamycin. Jour. Antibiot., Ser. A 18 (2): 101–103.

ILLUSTRATIONS

PLATE 1

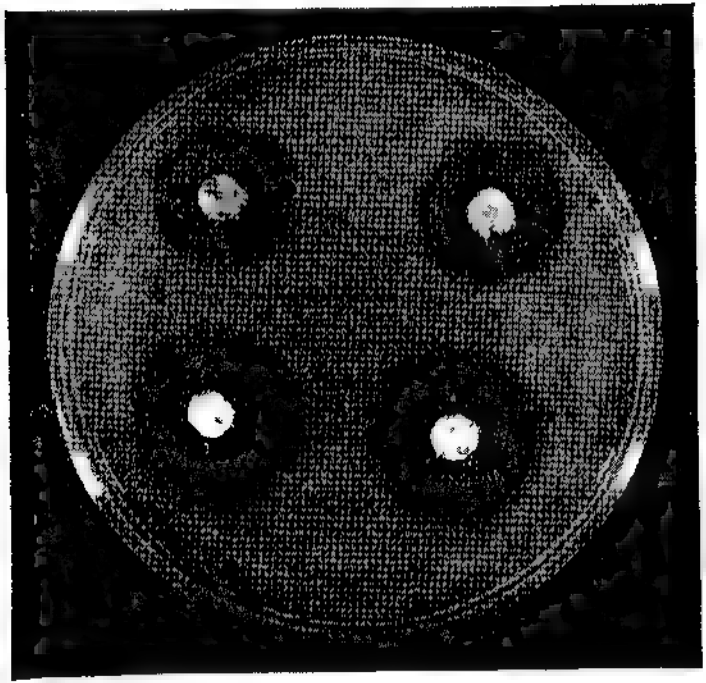
FIG. 1. Showing inhibition zones as clear areas around the agar plugs of Streptomyces isolates against *X. oryzæ*.

2. Showing inhibition zones as clear areas around the agar plugs of Streptomyces isolates against *P. oryzæ*.

PLATE 2

FIG. 1. Showing inhibition zones as clear areas around the paper discs dipped in culture brew of Streptomyces isolates against *X. oryzæ*.

2. Showing inhibition zones as clear areas around the paper discs dipped in culture brew of Streptomyces isolates against *P. oryzæ*.



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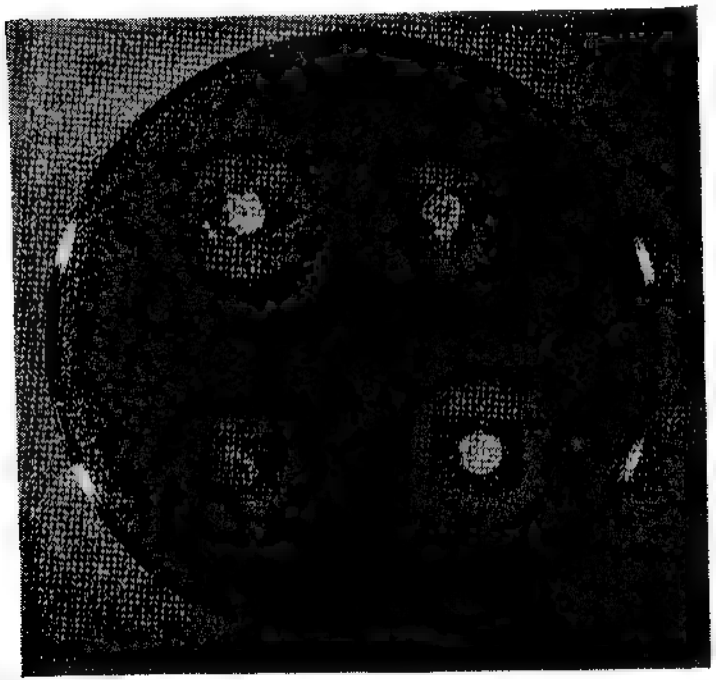

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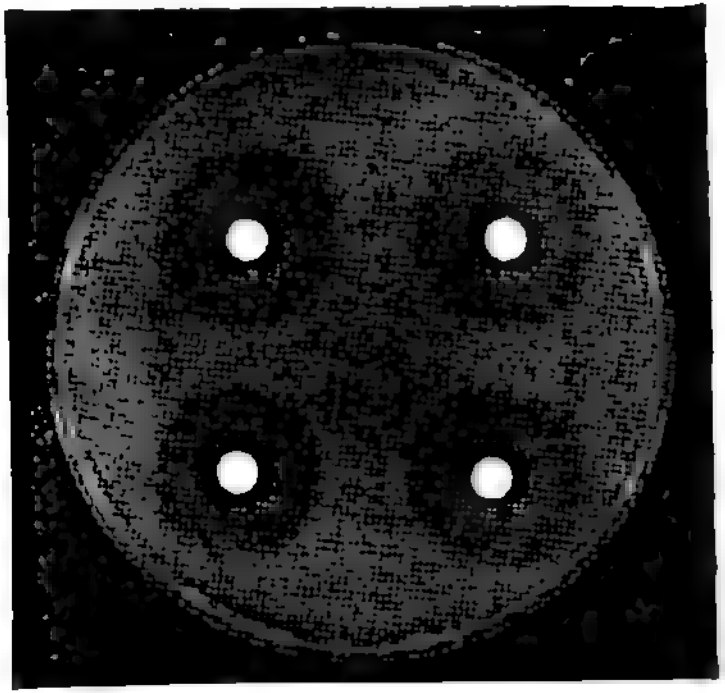
PLATE 1.



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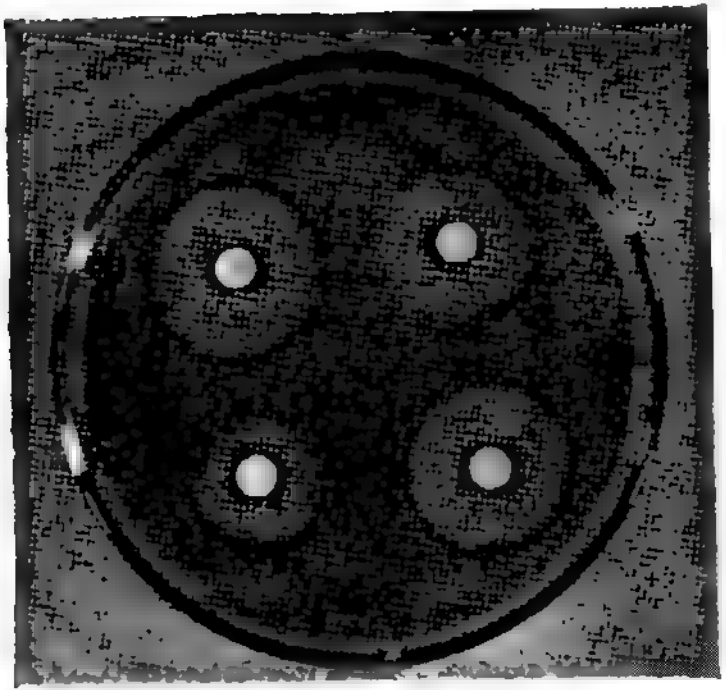

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PLATE 2.

CLINICAL EVALUATION OF NIST-PRODUCED ALLERGENIC EXTRACTS.

PART II. HYPOSENSITIZATION INJECTION TREATMENT WITH POLLEN EXTRACTS

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ABSTRACT

Hyposensitization injection treatment for at least a year with yard grass [*Eleusine indica* (L.) Gaertn.], amorsecos (*Andropogon aciculatus* Retz.), alabang X [*Dicanthium aristatum* (Poir) C.E. Hubb.], bermuda grass [*Cynodon dactylon* (L.) Pers.], and urai weed (*Amaranthus spinosus* Linn.) was undertaken in 22 patients with allergic perennial or nonseasonal rhinitis and/or nonseasonal asthma. Significant improvement was noted in 74 per cent rhinitis patients and in 69 per cent of asthmatic patients. Except for a few unpleasant symptoms due to intolerance to the dose concentration in a few patients, no further untoward reactions were noted from the prolonged use of these NIST prepared allergenic extracts.

In a previous study (Dacanay and Artiaga, 1969) it was reported that skin tests done on 120 individuals with allergic respiratory disease using pollen extracts prepared in the National Institute of Science and Technology from 22 of the most abundant and widely distributed grasses and weeds in the greater Manila area revealed that more than half of the test subjects gave positive skin reactions to yard grass [*Eleusine indica* (L.) Gaertn.], amorsecos (*Andropogon aciculatus* Retz.) alabang X [*Dicanthium aristatum* (Poir.) C. E. Hubb.], carabao grass (*Paspalum conjugatum* Berg.), bermuda grass [*Cynodon dactylon* (L.) Pers.], crab grass (*Digitaria* sp.), and urai weed (*Amaranthus spinosus* Linn.). As a result of this observation, the present study was started with the objective of determining the therapeutic value of the aforementioned extracts in such patients and to note, at the same time, if there may be untoward reactioons or effects arising from the long-term use of such extracts.

This report deals with observations made on 22 patients with allergic asthma and/or allergic rhinitis who had given positive skin reactions and subsequently received one year

hyposensitization injection treatment with NIST-prepared extracts of yard grass, amorsecos, alabang X, bermuda grass, and urai weed. These five extracts were selected among the seven previously mentioned which gave the most number of positive tests mainly because they are the most abundant plants in the Manila area. Recent investigations had also shown that these plants were among those which gave the largest number of pollen per floret and had high total rating of pollen production (Manalo and co-workers, 1969).

MATERIALS AND METHODS

Pollen extracts.—Extracts from the pollen of four grass species and one weed specie were used for skin testing and hyposensitization injection treatment. These consisted of the following: yard grass [*Eleusine indica* (L.) Gaertn.], amorsecos (*Andropogon aciculatus* Retz.), alabang X [*Dicanthium aristatum* (Poir.) C.E. Hubb.], bermuda grass [*Cynodon dactylon* (L.) Pers.], and urai weed (*Amaranthus spinosus* Linn.).

The concentrated extracts were prepared according to the method described by Laserna *et al* (1966). Coca's solvent [1] was used for extraction and Evans' buffered saline solution [2] used as diluent for the intradermal skin test and hyposensitization treatment solutions.

The diluting fluid is made by mixing one part of Solution I, one part of Solution II, and eight parts of distilled water.

Test subjects.—The 22 patients consisted of 14 females and 8 males, with ages ranging from 4 8 12 to 64 years. Sixteen patients had combined perennial allergic rhinitis and asthma,

[1] Coca's alkaline extracting fluid (Vaughan and Black, 1954)

NaCl	5.00 g
$NaHCO_3$	2.75 g
Phenol	4.00 cc
Distilled water to make 1000 cc	

[2] Evans' buffered saline (Vaughan and Black, 1954)

Stock solution No. I

NaCl	50.00 g
KH_2PO_4	3.63 g
$Na_2HPO_4 12H_2O$	14.31 g
Distilled water up to 1000 cc	

Stock solution No II

Carbolic acid, 4 per cent

three had perennial allergic rhinitis, and three patients had asthma only. Duration of rhinitis ranged from 3 months to 33 7/12 years (average—13 8/12 years) while that of asthma ranged from 5 months to 21 7/12 years (average—10 7/12 years) before admission to the study. Fifteen patients also had a past or concomitant history of skin allergies.

Clinical diagnosis of allergic respiratory disease was determined from the patient's history and physical examination supported by the finding of blood and/or nasal eosinophilia, a positive allergic family history, and positive skin tests to the pollen extracts under study. These patients also gave positive skin tests to house dust. Chest x-ray was also done to eliminate the presence of other nonallergic pulmonary diseases. In case of blood eosinophilia, feces examination was done to rule out the presence of parasitic infestation as a cause of eosinophilia. Scratch tests using the pollen concentrates and intradermal tests using 10 PNU [3]/ml and 100 PNU/ml dilutions of the pollen extracts were done on each patient. Post-treatment blood examination, nasal smear and skin tests were also made on each patient.

Pulmonary function tests using the McKesson-Vitalor (Model VC–25A) to determine the forced vital capacity for the first, second ($FVC_{1.0}$) and maximum expiratory flow rate (MEFR) were done on each asthmatic patient before and after hyposensitization treatment. The best of three trials was recorded for each patient.

Hyposensitization treatment procedure.—A mixture of the five aforementioned pollen extract materials was prepared from their pollen concentrates with Evans buffered solution as diluent. This material was injected hypodermically into each patient generally at 1-week interval, using a starting dose of 0.025 to 0.05 ml of a 5×10^{-3} to 5×10^{-8} PNU/ml dilution depending on the sensitivity of the patient, with weekly increments of 0.025 to 0.05 ml. After reaching a dose of 0.225 to 0.25 ml, the solution was changed to the next higher one of ten-fold strength and weekly increments given as before. This procedure was followed for as long as the dosage increase was tolerated until the maintenance dose was reached and the patient had received at least 1 year of injection treatment. None of these patients received dust injection treatment before and during the period of pollen injection therapy.

[3] 1 PNU = 0.00001 mg protein nitrogen.

Assessment of treatment results.—This was based on the following: (*a*) an evaluation of the weekly progress forms accomplished by each patient indicating the amount of daily medications taken and other treatment, personal observation of daily condition and other pertinent information regarding limitation or restriction of activities like absence from school or work, hospitalization, etc. Each patient was told to continue with the usual environmental and dietary control measures, as well as the symptomatic medications taken whenever necessary, previous to his inclusion in the study.

A score system was devised in order to make it more convenient for the observer to quantify numerically the patient's progress in terms of frequency and severity of symptoms as well as medication requirements. The symptom score used was as follows: three points for each severe asthmatic attack, two points for a moderate one, and one point for a slight attack. For the nasal symptoms, three points each was given for severe rhinitis, stuffiness or sneezing, two points each for moderate, and one point each for a slight degree of these nasal complaints. The medication score used was as follows: five points for each steroid tablet taken, four points each for use of oxygen, intravenous fluid or an injection of adrenalin or bronchodilator preparation, three points for each bronchodilator tablet, capsule or per average dose in liquid form, two points for each use of bronchodilator nebulizer (aerosol), one point for each antihistamine tablet, capsule, or average dose of liquid. The total symptom score for the first 6 months or first half of treatment was obtained and compared with the total score for the second half of treatment. The resulting percentage difference was computed and rated as follows:

\+ or — 1 to 33 per cent = slight increase or decrease
\+ or — 34 to 66 per cent = moderate increase or decrease
\+ or — 67 to 99 per cent = marked increase or decrease
\+ or — 100 per cent or over = very marked increase or decrease.

The same method of rating was applied to the medication scores.

(*b*) the results of the weekly interview and observations made on the patient's physical condition.

(*c*) changes in the pulmonary function tests taken before and after treatment in the case of asthmatic patients.

(*d*) a comparison of the results of the pre- and post-treatment skin tests.

RESULTS

In the analysis of results, it was found more convenient to divide the 22 patients into two groups—the first group with rhinitis and the second group with asthmatic symptoms. Sixteen patients with both asthma and rhinitis, therefore, were included in both groups (Patients No. 1–16, Tables 1–4). This was done because some of these patients did not show a direct correlation in improvement of both complaints. In comparing the progress of each patient between the first and second half of the treatment period, each patient, in a sense, became his own control.

TABLE 1.—*Summary of over-all evaluation of 19 rhinitis patients after treatment.*

Degree of improvement	Number of patients	Percentage	Total
		Per cent	*Per cent*
Very marked improvement	4	21	
Marked improvement	1	5	
Moderate improvement	3	16	74
Slight improvement	6	32	
No improvement	5	26	26
Total	19		100

TABLE 2.—*Summary of over-all evaluation of 19 asthma patients after treatment.*

Degree of improvement	Number of patients	Percentage	Total
		Per cent	*Per cent*
Very marked improvement	2	11	
Marked improvement	5	26	
Moderate improvement	3	16	69
Slight improvement	3	16	
No improvement	6	31	31
Total	19		100

TABLE 3—*Relationship between symptom score, medication score and post-treatment condition in rhinitis cases.*

Patient	Diagnosis	Symptom score[2] (Per cent difference 1st & 2nd half of Rx)	Medication score[2] (Per cent difference 1st & 2nd half of Rx)	Evaluation of post-Rx condition
1	PR[1], severe	Marked decrease (90.5)	Slight increase (+16)	Moderate improvement
2	do	Very slight decrease (9.6)	Very marked decrease (—100)	Slight improvement
3	PR, moderate	Very marked decrease (—100)	do	Very marked improvement
4	do	do	Marked decrease (—78)	Marked improvement
5	do	Slight decrease (25)	Very marked decrease (100)	Slight improvement
6	do	Slight increase (+7.1)	do	No improvement
7	do	Slight decrease (—10.7)	Moderate increase (+37)	do
8	PR, slight	Very marked decrease (100)	Very marked decrease (100)	Very marked improvement
9	PR, moderate	do	Slight increase (+21)	Slight improvement
10	PR, slight	do	Very marked decrease (—100)	Very marked improvement
11	PR, moderate	Slight decrease (—11)	do	Slight improvement
12	do	Very marked decrease (—100)	do	Very marked improvement
13	do	Moderate decrease (41)	Moderate decrease (—64)	Moderate improvement
14	do	Very marked increase (+170)	Marked decrease (—81)	No improvement
15	do	Slight decrease (9)	Moderate decrease (—64)	Slight improvement
16	do	Very marked increase (+116)	No increase (0)	No improvement
17	PR, severe	Slight decrease (—9.5)	Very marked increase (+764)	do
18	do	Slight decrease (—21.4)	Slight decrease (—18)	Slight improvement
19	PR, moderate	Moderate decrease (61.9)	Marked decrease (—70)	Moderate improvement

[1] Perennial rhinitis

[2] Rating in symptom/medication score:

1—33 per cent = Slight increase (+) or decrease (—)

34—66 per cent = Moderate increase (+) or decrease (—)

67—99 per cent = Marked increase (+) or decrease ( )

100 Per cent or above = Very marked increase (+) or decrease ( )

TABLE 4.—*Relationship between starting dose, total dose received (PNU), and post-treatment condition in rhinitis cases.*

Patient	Diagnosis	Starting dose (PNU/ml)	Total dose (PNU)	Evaluation of post-R_x condition
1	PR [1], severe	5×10^{-3} (a)	.00287	Moderate improvement
2	do	5×10^{-4} (b)	.0048	Slight improvement
3	PR, moderate	5×10^{-5} (c)	.000023	Very marked improvement
4	do	5×10^{-6}	.00002	Marked improvement
5	do	do	.009894	Slight improvement
6	do	do	.004773	No improvement
7	do	do	.03312	do
8	PR, slight	5×10^{-5}	.507e2	Very marked improvement
9	PR, moderate	5×10^{-6}	.0089113	Slight improvement
10	PR, slight	do	.036265	Very marked improvement
11	PR, moderate	do	.046871	Slight improvement
12	do	5×10^{-8}	.000065	Very marked improvement
13	do	do	.0000033	Moderate improvement
14	do	5×10^{-7}	.0001404	No improvement
15	do	do	.0010013	Slight improvement
16	do	do	.000021	No improvement
17	PR, severe	5×10^{-3}	26.158	do
18	do	5×10^{-8} (d)	.0000053	Slight improvement
19	PR, moderate	5×10^{-6} (e)	.00000376	Moderate improvement

[1] PR = Perennial or nonseasonal rhinitis

(a) = Starting dose changed to 5×10^{-4} PNU/ml due to numbness of extremities and feeling of warmth after 1st dose

(b) = Starting dose changed to 5×10^{-5} PNU/ml due to very marked local reaction

(c) = Starting dose changed to 5×10^{-6} PNU/ml due to dizziness and feeling of warmth after 1st dose

(d) = Starting dose changed to 5×10^{-8} PNU/ml due to choking sensation, increased rhinorrhea and lacrimation after 1st dose

(e) = Starting dose changed to 5×10^{-8} PNU/ml due to dizziness shortly after 1st injection

Rhinitis.—Table 1 is a summary of the over-all evaluation made on the 19 patients with nonseasonal or perennial rhinitis whose symptom and medication ratings based on the percentage difference between their total symptom and medication scores for the first and second half of the treatment period are seen on Table 3. In this group, 14 patients (74 per cent) showed improvement in symptoms, though of varying degrees; four patients showed very marked improvement, one showed marked improvement, three were moderately improved, while six were only slightly improved. Five patients (26 per cent) showed no improvement at all.

Total treatment received in protein nitrogen units (PNU) ranged from 3.76×10^{6} PNU to 26.158 PNU (Table 4).

Skin testing on these patients after the treatment period showed a decreased reaction of at least 10 to a hundredfold strength in 63 per cent of the total skin tests done, using the five pollen extracts under study.

Asthma.—Of the 19 patients with perennial or nonseasonal asthma, analysis based on their symptom and medication ratings (Table 5) showed that thirteen (69 per cent) had improved after 1 year of treatment. Very marked improvement was noted in two, marked improvement in five, moderate improvement in three, and slight improvement in another three patients. There were six patients (31 per cent) who remained unimproved (Table 2).

Total treatment dose received by this group ranged from 3.3×10^{6} PNU to 0.60752 PNU (Table 6).

Results of the pre- and post-treatment pulmonary function tests done on each patient; namely, the forced expiratory volume for the 1st, second ($FEV_{1.0}$) and maximum expiratory flow rate (MEFR) are seen in Table 5. Ten of the patients showed slight to very marked increase in both $FEV_{1.0}$ and MEFR. Thirteen patients showed an increase while six showed a decrease in $FEV_{1.0}$. In the MEFR measurements, 13 patients had an increase, four patients showed a decrease while the measurement could not be done in two children due to technical difficulties.

Post-treatment skin tests using the five pollen extracts on the asthmatic patients showed a decrease of 10 to a hundredfold strength in 67 per cent of the total skin tests made.

TABLE 5.—*Relationship between symptom score medication score, pulmonary function tests and post-treatment condition in asthma cases.*

Patient	Diagnosis	Symptom score[2] (Per cent difference 1st & 2nd half of Rx)	Medication score[2] (Per cent difference 1st & 2nd half of Rx)	Pulmonary function: FEV$_{1.0}$ Per cent diff. pre- & post Rx	Pulmonary function: MEFR Per cent diff. pre- & post Rx	Evaluation of post-Rx condition
1	PA[1], moderate	Moderate increase (+60)	Moderate increase (+41)	+37.7	+12	No improvement
2	do	Marked decrease (—77)	Marked decrease (—83)	+11.5	+81	Marked improvement
3	PA, severe	Marked decrease (—83)	[illegible] decrease (—76)	+17.0	+127	do
4	do	Marked decrease (—67)	Marked decrease (—88)	— 0.0	+7.0	do
5	PA, moderate	Very slight increase (+1)	Moderate increase (+34)	— 0.33	+15	No improvement
6	do	Moderate decrease (—66)	Marked decrease (—72)	+72.8	+169	Moderate improvement
7	do	Very marked decrease (—100)	Very marked decrease (—100)	+11.7	+34	Very marked imp.
8	PA, slight	do	do	+ 1.4	+15	do
9	PA, moderate	do	Moderate decrease (—36)	—21.0	—34	Moderate improvement
10	do	Marked decrease (—80)	Slight decrease (—29)	— 8.0	—15	Slight improvement
11	do	Marked decrease (—93)	Slight decrease (—31)	+64	Not done	Marked improvement
12	PA, severe	Slight decrease (—19)	Slight decrease (—7)	+96.0	+343	Slight improvement
13	PA, moderate	Moderate decrease (—49)	Moderate increase (+30)	—7	—26	No improvement
14	do	Moderate decrease (—62)	Moderate increase (—66)	[illegible]90	Not done	Moderate improvement
15	do	Slight decrease (—33)	Very slight decrease (—4)	+11.0	+34.0	Slight improvement
16	do	Marked increase (+83)	Very marked increase (+704)	+ 3.0	0.5	No improvement
17	PA, severe	Slight increase (+25)	Moderate increase (+37)	+21.1	+91.0	do
18	PA, slight	Marked decrease (—85)	Very marked decrease (—100)	— 1.0	+31.0	Marked improvement
19	PA, moderate	Marked increase (+81)	Moderate decrease (—34)	+24.0	+29.0	No improvement

[1] Perennial Asthma

[2] Rating in symptom/medication score:

1—33 Per cent = Slight increase (+) or decrease (—)

34—66 per cent = Moderate increase (+) or decrease (—)

67—99 per cent = Marked increase (+) or decrease (—)

100 Per cent and above = Very marked increase (+) or decrease (—)

Aside from the previously mentioned five pollens, other pollens to which there was a significant number of positive skin reactions before treatment were as follows: carabao grass (*Paspalum conjugatum* Berg.) which was positive in 18 patients, crab grass (*Digita ia species*) positive in 14 patients, kogon [*Imperata cyandrica* (L.) Beauv.] positive in 13 patients, and talahib [*Saccharum spontaneum* (L.) subsp. *indicum* Hack.] positive in 10 patients Although these pollens were not included in the specific treatment, it was noted that after 1 year observation period, a ten to a hundredfold decrease in skin reactivity occurred in the 10 patients previously positive to talahib, in 12 of the 13 patients previously positive to kogon, in six of the 14 patients previously positive to crab grass and in nine of the 18 patients previously positive to carabao grass.

In the treatment of both groups, strength of starting dose was based on the results of the skin test reactions in individual patients with the ones showing marked skin reactions started on more dilute strengths.

Eleven of the patients were started on 5×10^{-6} PNU ml However, three of these had to be shifted to a solution a hundredfold weaker because of untoward reactions (Tables 4 and 6). One patient with rhinitis showed increased rhinorrhea and lacrimation with a choking sensation. The second patient complained of dizziness a few minutes after receiving the first dose, while the third one showed a marked local reaction of erythema, 5 inches in diameter with a moderate sized wheal. Two patient who started on a dose of 5×10^{-4} PNU, ml had to be changed to a weaker treatment solution because one complained of slight dizziness and increased warmth while the second patient showed very marked local reaction. Of two other patients started on a dose of 5×10^{-3} PNU/ml, one complained of increase warmth and some numbness of the extremities on receiving the first treatment dose. One patient was started on 5×10^{-7} PNU ml, three on 5×10^{-7} PNU ml, and three others on 5×10^{-5} PNU/ml. All the patients who had shown untoward reactions at onset of treatment and consequently had to be shifted to much weaker treatment mixtures did not complain further of unpleasant symptoms as progressive increases in the amount and strength of their doses were made in the course of treatment.

TABLE 6.—*Relationship between starting dose, total dose received (PNU), and post-treatment condition, in asthma cases.*

Patient	Diagnosis	Starting dose (PNU/ml)	Total dose (PNU)	Examination of post-R_x condition
1	PA [1], moderate	5×10^{-8}(a)	.00287	No improvement
2	do	5×10^{-4}(b)	.0048	Marked improvement
3	PA, severe	5×10^{-6}(c)	.000023	do
4	do	5×10^{-6}	.00002	do
5	PA, moderate	do	.009894	No improvement
6	do	do	.004773	Moderate improvement
7	do	do	.03312	Very marked improvement
8	PA, slight	5×10^{-5}	.60752	do
9	PA, moderate	5×10^{-6}	.0059113	Moderate improvement
10	do	do	.036265	Slight improvement
11	do	do	.046571	Marked improvement
12	PA, severe	5×10^{-5}	.000065	Slight improvement
13	PA, moderate	do	.0000033	No improvement
14	do	5×10^{-7}	.0001404	Moderate improvement
15	do	do	.0010013	Slight improvement
16	do	do	.000021	No improvement
17	PA, severe	5×10^{-6}(d)	.0000287	do
18	PA, slight	5×10^{-6}	.0808032	Marked improvement
19	PA, moderate	5×10^{-5}	.007365	No improvement

[1] PA Perennial or nonseasonal asthma.
(a) = Starting dose changed to 5×10^{-4} PNU/ml due to numbness of extremities and feeling of warmth after 1st dose.
(b) = Starting dose changed to 5×10^{-5} PNU/ml due to very marked local reaction.
(c) = Starting dose changed to 5×10^{-6} PNU/ml due to dizziness and feeling of warmth after 1st dose.
(d) = Starting dose changed to 5×10^{-8} PNU/ml due to very marked local reaction.

Other observations.—Nasal eosinophilia was observed in 15 out of the 22 patients before treatment; 14 of these patients remained positive while two patients with negative pretreatment findings became positive after treatment.

Blood eosinophilia was found in 14 out of the 22 patients prior to treatment. After 1 year of treatment, 12 of them remained positive while two patients became negative. However, of the eight patients with previous negative findings, three became positive, thus giving a total of 15 patients with blood eosinophilia after treatment.

DISCUSSION

Although Curtis (1900) had advocated the injection of progressively increasing doses of pollen antigen as a treatment of hay fever or seasonal rhinitis, this form of treatment only gained popular acceptance and widespread use after Noon and Freeman (1911) had done further observations and work on it, utilizing the procedure effectively in the treatment of pollenosis and also proposing a standard unit for the allergens used in the treatment. The procedure is also sometimes referred to as hyposensitization, desensitization or immunization injection treatment. Apart from complete elimination or withdrawal of an offending allergen which is often very difficult to do especially in the case of inhalant allergens, this treatment has remained the most effective method used in the care of allergic respiratory diseases, especially of the seasonal type. However, it is also used in other forms of allergic diseases like insect allergy, certain forms of allergic contact dermatitis especially from plants like poison ivy, etc.

Injection treatment is specific and involves the production of certain immunologic changes in the treated individual. It has been found by several workers (Levine and Coca, 1926; Sherman, 1941) that during the first few months of injection treatment, there is an increase in the amount of reaginic or skin-sensitizing antibody while another antibody called the blocking antibody becomes demonstrable in the serum. (Cooke *et al*, 1935). As treatment continues, the reagins tend to decrease but the blocking antibodies continue to be found in the serum. Although it has been observed that symptoms tend to improve with a decrease of skin-sensitizing antibody or reagin, increased

amounts of blocking antibody have been found in patients whose symptoms were less severe than what might have been expected from their reagin titers. This observation has led to the conclusion that the blocking antibody exerts protective action—by competing with the skin-sensitizing antibody and binding the antigen. However, the exact protective mechanism involved is still not fully understood.

Several workers have reported that hyposensitization injection treatment gives good results ranging from 70 per cent to as much as 90 per cent in patients with hay fever (Vender Veer, 1947; Rapaport and Schaffer, 1963). In case of multiple allergies, the results seem to be somewhat less effective. In this study, 74 per cent of our patients with perennial rhinitis showed varying degrees of improvement. However, no direct correlation was found between the total dose received and the degree of improvement among our rhinitis patients. For example, patient No. 17, who received the largest amount of PNU (26.158 PNU), showed no improvement of symptoms while patient No. 3 showed very marked improvement on a total dose of 2.3×10^5 PNU. We have not had a clear-cut case of seasonal pollenosis or asthma as pollen season in this area extends through the greater part of the year with peaks occurring generally in late October to January (Payawal and Laserna, 1966) but can extend through February or early March (Remo, 1970). Our patients with perennial or nonseasonal asthma showed 69-per cent improvement on injection treatment with the NIST-prepared allergenic extracts. As in the rhinitis group, no direct correlation was observed between the total number of PNU received and the degree of improvement in the patients. In the overall evaluation of the post-treatment condition of the asthmatic patients, more emphasis was placed on the symptom and medication scores which covered the whole period of treatment rather than on the results of the pulmonary function test taken on two occasions, pre- and post-treatment, particularly if the latter findings did not seem to support the significant improvement noted from the symptom and medication record. In comparing the patients' subjective evaluation of their post-treatment condition to that of the medical observer's, there was agreement only in 10 out of 19 patients with rhinitis (53-per cent correlation) and in 8 out of 19 patients with asthma (42-per cent correlation).

Although our patients were positive to the five pollens used in the treatment, they had shown positive skin test reactions to several other pollens not included in the treatment and had also reacted to dust allergen. Most probably, the percentage of improvement would increase if: (*a*) treatment against the other significantly involved allergens were given; (*b*) the treatment were continued for a longer period as has been found in a greater number of patients followed beyond the first year of injection treatment (Lowell and Franklin, 1963); and or (*c*) total treatment doses were much higher. We had veered towards the low dose treatment in our study because of some unpleasant reactions that were encountered, as mentioned previously, in some of our patients when treatment was started. However, we feel that in spite of this, our results with the use of extracts produced locally at NIST are quite comparable to the reported observations regarding effectiveness of injection treatment.

Evidence of decreased skin reactivity with increased tolerance for injections has also been found in patients who had received treatment for several years. Changes in skin tests were directly correlated with the amount of treatment dose used, higher treatment doses producing marked decrease in skin reactivity (Sprecace *et al*, 1966). In our study, 63 per cent of the skin tests in patients with perennial rhinitis and 67 per cent of the skin tests in asthmatic patients using the five pollens under study showed a ten to a hundredfold decrease in their reactivity after a year of treatment. According to these workers, such a reduction in skin reactivity seems to show that specific injection treatment can produce a decrease in sensitization of the treated individual.

An interesting observation in the post-treatment skin test results in our study group was the finding of decreased skin reactivity in a significant number of the previously positive skin test reactions particularly to talahib and kogon grasses although no specific treatment for these had been given. An intriguing thought is the possibility of a common or closely related antigen being present in these local pollens which were studied so that a patient who has reacted to one pollen can also react to the others. Such antigenic similarity has been found in the pollen of five principal grasses in the northern U. S. (Sherman, 1968). This possibility, as far as our local grass pollens are concerned, can only be determined by immunochemical and further clinical studies on these pollens.

SUMMARY

In 22 patients with allergic perennial or nonseasonal rhinitis and/or nonseasonal asthma, injection treatment for at least a year with NIST-produced extracts of yard grass, bermuda grass, alabang x, amorsecos, and urai weed gave significant improvement in 74 per cent of rhinitis patients and 69 per cent of asthmatic patients. Except for some unpleasant symptoms encountered by some patients which may be explained by an intolerance to the concentration of the dose used at the onset of treatment, no further untoward reactions were noted from the prolonged use of these extracts. Reduction of skin reactivity was noted in 63 per cent of total skin tests done in the rhinitis patients and in 67 per cent of total skin tests done in the asthmatic patients.

A brief review of the literature regarding the efficacy and the possible mechanism of protection produced by injection treatment with allergenic extracts is also presented.

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REFERENCES

COOKE, R. A., J. H., BARNARD, S. HEBALD, and STILL (1935) Serological evidence of immunity with coexisting sensitization in a type of human allergy, hay fever. Jour. Exp. Med. 62: 733.

CURTIS, H. H. (1900). The immunizing cure of hay fever. Med. News 77: 16. Cited in Sherman, W. B. (1968), Hypersensitivity). Mechanisms and Management. Philadelphia: W. B. Saunders Co., p. 166.

DACANAY, E. P., and L. M. ARTIAGA (1969). Clinical evaluation of NIST-produced allergenic extracts. I. Skin testing with pollen extracts. (Grasses and weeds). Philip. Jour. Sci. 98: 235–246.

FREEMAN, J. (1911). Further observations on the treatment of hay fever by hypodermic inoculations of pollen vaccine. Lancet 2: 814. Cited in Sherman, W. B. (1968). Hypersensitivity. Mechanisms and Management. Philadelphia: W. B. Saunders Co., p. 237.

LASERNA, G., and J. B. MANALO (1966). Preparation and standardization of allergenic extracts of local air-borne pollen grains. Philip. Jour. Sci. 95: 275–280.

LEVIN, P., and A. F. COCA (1926). Studies in hypersensitiveness. Nature of alleviating effect of specific treatment of atopic condition. Jour. Immun. 11: 449.

LOWELL, F. C., and W. FRANKLIN (1963). A "double-blind" study of treatment with aqueous allergenic extracts in cases of allergic rhinitis. Jour. Allergy 34: 165–182.

MANALO, J. B., P. C. PAYAWAL, and G. LASERNA (1969). Pollen production of some allergenic plants in the Manila area. Philip. Jour. Sci. 98: 11 15.

NOON, L. (1911). Prophylactic inoculation against hay fever. Lancet 1: 1572. Cited in Sherman, W. B. (1968), Hypersensitivity. Mechanisms and Management. Philadelphia: W. B. Saunders Co., p 237.

PAYAWAL, P., and G. LASERNA (1966). Aeropalynological studies at Manila, 1963. Philip. Jour. Sci. 95: 171–187.

RAPAPORT, H. G., and N. SCHAFFER (1963). Treatment of inhalant allergy. From Speer, F. (Ed.) (1963). The allergic child. New York, Evanston and London: Harper and How, Publishers, pp. 466–483.

REMO, I. (1970). Personal communication.

SHERMAN, W. B. (1941). Changes in serologic reaction and tissue sensitivity in hay fever patients during the early months of treatment. Jour. Immun. 40: 289.

SHERMAN, W. B. (1968). Hypersensitivity. Mechanisms and Management. Philadelphia: W.B. Saunders Co. p. 230.

SPRECACE, G. A., S. G. POMPER, W. B. SHERMAN, A. LEMLICH, and H. ZIFFER (1966). The effect of antigen injections on skin reactivity to antigents. Jour. Allergy 38: 9–25.

VANDER VEER, A. Hay fever. In Cooke, R.A. (Ed.) (1947). Allergy in Theory and Practice. Philadelphia: W.B. Saunders Co., Chap. 10, Sec. III.

VAUGHAN, W. T., and J. H. BLACK (1954). Practice of Allergy. 3rd Ed. St. Louis: C. V. Mosby Co., p. 268.

IMPROVEMENT OF THE DRYING PROPERTY OF LUMBANG OIL. I. FORMATION OF UREA COMPLEXES

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ABSTRACT

The drying property of lumbang oil has been improved with a maximum increase of 1[illegible] iodine number units by the formation of urea complexes. Studies on solvent effect show that ethanol (95 per cent) gives the same effect as methanol and isopropyl alcohol while the oil: urea molar ratio has been established at 1:1.

INTRODUCTION

Lumbang oil or candlenut oil is obtained from the nut of the tree *Aleurites moluccana* (Linn.) Willd., a plant indigenous in the country. At present, the oil is used as drying oil in paint manufacture and in tempering of hardwood in the lumber industry. Investigations made on its drying properties, rate of polymerization and jelling properties have shown that it is relatively poor when compared with commonly used imported drying oils such as tung oil, linseed oil, and oiticica oil (Zimmerchied *et al*, 1950).

The glyceride of lumbang oil is composed mostly of unsaturated fatty acids (Bailey, 1951a):

	Per cent
oleic acid	10.5
linoleic acid	48.5
linolenic acid	28.5
saturated fatty acids	12.5

Although this oil contains considerable amount of unsaturated fatty acids, it is not unsaturated enough to be effectively used as a drying oil or in oil-tempering of hardwood. Its degree of unsaturation must have to be sufficiently increased in order to improve its performance as a drying oil. In this connection, several methods are possible such as fractional crystallization, fractional distillation, segregation, or catalytic isomerization (Mills, 1952; Switzer, 1952).

One of the methods investigated to enhance the drying properties of lumbang oil is by the use of urea complexes.

The formation of urea complexes was first shown by Bengen (1940) who separated straight chain compounds from cyclic compounds by complex formation and was later confirmed by Zimmerchied *et al* (1950) and Schlenk and Holman (1950). This principle was later applied on the separation of natural linoleic (cis, cis) and linolenic acids (66–95 per cent) from oils rich in these acids, and by Swern and Parker (1953), who made use of the preferential precipitation of urea complexes of saturated and monounsaturated acids from corn oil fatty acids and preferential precipitation of saturated, monounsaturated and diunsaturated acids from linseed oil fatty acids or perilla oil fatty acids.

Recently, Eisenhauer and Beal (1968), separated cyclic fatty acids from straight chain fatty acid by urea adduct formation. Among others, the advantages mentioned were that urea adduct formation avoids hydrogenation and low-temperature crystallization and thus, unsaturated cyclic and unsaturated straight chain products could be recovered as individual fractions.

Although several investigations have been done (as mentioned above) dealing on urea complexes of fatty acids or fatty esters, no study has been undertaken on the application of urea complexes directly on the oil itself. The oil would be relatively cheaper and more practical as a starting material than the fatty acid. The process which is also known as "segregation" is based on the theory that the fatty acids composing the oil are randomly distributed thus making it possible to have a combination of the most unsaturated fatty acids in the glyceride (Hilditch, 1956). This principle has been successfully applied on an industrial scale especially on liquid-liquid segregation using furfural (Mills, 1952; Switzer, 1952).

EXPERIMENTAL

Materials.—1. Commercial lumbang oil was used in this study. This was supplied by the Philippine Wallboard Corporation (Nasipit Lumber Company, Inc.) and the YCO Paint Company, Philippines.

2. 'Laboratory reagent' urea was obtained from the British Drug House, Ltd., England; 'analytical reagent' ethyl ether from Mallinckrodt Chemical Works, U.S.A.; "pro analyse"

methanol from Riedel-De Haën AG, Seelze, Germany; 95 per cent ethanol from Belman Laboratories, Philippines; and isopropyl alcohol from Golden Bat Enterprises, Philippines.

3. Nitrogen gas was purchased from the Superior Gas Company, Philippines.

Equipment. –For refractive indices, Abbe' Refractometer was used.

Procedure. –A weighed amount of lumbang oil was dissolved in a hot solution composed of a weighed amount of urea in the solvent (metnanol, ethanol, or isopropyl alcohol). The solution was further heated for about 5 minutes, after which the mixture was cooled to room temperature and the solid adduct which crystallized, filtered through a Buchner funnel. The filtrate which contained the unsaturated portion was extracted with diethyl ether and washed with water to remove the urea crystals. The ether solution was then evaporated under a flush of nitrogen and the resulting oil (extract) was subjected to analysis.

To the urea adduct was added distilled water in order to break the complex and liberate the saturated portion of the oil (raffinate). This was facilitated by the addition of ether which dissolved the oil, followed by the removal of ether by nitrogen flush.

All analyses were conducted as described by the standard AOCS methods.

RESULTS AND DISCUSSION

By complexing lumbang oil with urea, the oil could be separated into two portions, one which is composed of the most unsaturated fatty acid glyceride (extract) and the other composed of the least unsaturated fatty acid glyceride (raffinate).

The results are all shown in Tables 1 to 3. The measure for the effectivity of the method is the relative increase in the iodine number of the resulting extract when compared with that of the original oil. Table 1 shows that the optimum molar ratio between the oil and urea should be 1:1 with an improvement of 18 iodine number units. Below this, i.e, 1 mole oil: $\frac{1}{2}$ mole urea, there is an improvement of only two iodine number units. However, above 1 mole urea: 1 mole oil ratio, the increase in iodine number is also about 18 units, but then, this would obviously be uneconomical.

Table 2 shows that the three solvents considered; namely, methanol, 95-per cent ethanol and isopropyl alcohol, gave practically identical results with an improvement of about 17 to 18 units. Based on this, 95-per cent ethanol was chosen because it is readily obtained.

TABLE 1.—*Effect of oil-urea ratio on the degree of unsaturation of the product using methanol as solvent.*

Oil-urea ratio (moles)	Iodine number		Increase in iodine number[2] (units)	Refractive index (25° C.)		Yield (Per cent)	
	Extract	Raffinate		Extract	Raffinate	Extract	Raffinate
1:0.5	150	135	2	1.4736	1.4733	76.0	17.[illegible]
1:1	166	159	18	1.4756	1.4748	74.8	2[illegible]
1:1.5	166	[illegible]9	18	1.4758	1.4731	66.[illegible]	2[illegible]
1:2	16[illegible]	155	17.5	1.4762	1.4749	71.0	11.1

[1] Tungao lumbang oil sample; iodine number, 148.

[2] This is obtained by subtracting the iodine number of original lumbang oil from that of the extract.

TABLE 2.—*Effect of solvent on the degree of unsaturation of the product using a 1:1 oil[1]-urea ratio.*

Solvent	Iodine number		Increase in iodine number units[2]	Refractive index (25°C.)		Yield (Per cent)	
	Extract	Raffinate		Extract	Raffinate	Extract	Raffinate
Methanol	166.0	159	18.0	1.4756	1.4748	74.8	20.0[illegible]
Isopropyl alcohol	164.5	162	16.5	1.4759	1.4744	76.0	8.1
Ethanol (95 per cent)	167.0	159	17.0	1.4759	1.4750	81.0	5.7

[1] Tungao lumbang oil sample; iodine number—148.

[2] Iodine number of extract—Iodine number of original lumbang oil

TABLE 3.—*Effect of multiple extraction on the degree of unsaturation of the products using 1:1 oil-urea ratio and ethanol as solvent.*

Number of extractions	Iodine number[1]		Increase in iodine number (units)[2]	Refractive index 25° C.		Yield (Per cent)	
	Extract	Raffinate		Extract	Raffinate	Extract	Raffinate
First	151.9	146	12.5	1.4765	1.4769	69.2	16.5
Second	154	145	13.0	1.4765	1.4760	68.3	17.2
Third	151	153	12.6	1.4760	1.4756	67.3	18.2

[1] Iodine number of lumbang oil sample—150 (from YCO Paint Company, Manila).

[2] Iodine number of extract—Iodine number of original lumbang oil.

The choice of the solvent in this study, considers the fact that the structure of the R-group in the alcohol (ROH) molecule must not be long as to react with urea, since the latter forms complexes with straight chain compounds (Zimmechied *et al*, 1950). The soivent must also possess a polar center (like the hydroxyl group of the alcohol) which could form a hydrogen bond with urea and hasten the suspension of the urea molecule for effective interaction with the oil. Thus the solvents considered were limited to methanol, ethanol and isopropyl alcohol. Alcohols such as n-butyl or n-amyl alcohol besides being difficult to obtain would be unsuitable because they possess long R-groups.

The refractive indices of the product show an increasing trend with increasing iodine number. This is in agreement with the findings that there is a direct proportionality relationship between the iodine number and refractive index (Bailey, 1951).

The results of the study on the multiple extraction of lumbang oil using 1 mole oil: 1 mole urea ratio (Table 1) and ethanol (95 per cent) as solvent (Table 2) are presented in Table 3. This shows that in general, an improvement of about 12 to 15 iodine number units could be achieved using urea complexes. This change could be attained immediately after the first extraction. In the second and third extractions, the alterations in the iodine number are still within the range of 12 to 15 units. This could only mean that there is a point beyond which further improvement in the iodine number is no longer possible. In other words, there is a ceiling or leveling off point, and for lumbang oil, this point is reached immediately after the first extraction with an increase of about 12 to 15 units.

This conclusion is also corroborated by the fact that if the iodine numbers of the extracts and of the raffinates are compared, it is noticed that the two become almost equal especially for the second and third extractions. Theoretically, the iodine number of the extract is supposed to be higher than that of the raffinate if the oil could still be separated. But when the leveling-off point is reached, it is no longer possible to obtain two fractions with different iodine numbers, because by now the composition of the glyceride shall have become almost uniform. Thus, the iodine numbers of the two fractions become almost equal.

The variation in results between the data in Tables 1 and 2 which give an increase of 18 iodine number units and those in Table 3 with 12 to 15 iodine number units is attributed to differences in the source of the sample. In the former (Tables 1 and 2) the sample used was "Tungao" lumbang oil from the Philippine Wallboard Corporation (Nasipit Lumber Company Inc., Nasipit, Agusan, Phil.) whereas for the multiple extraction study, because large amounts were needed, the oil was supplied by YCO Paint Company, Tanduay St., Manila, Philippines. Differences in purification treatments might account for the differences between the two sets of results.

The results obtained in this study are in general agreement with those obtained in the extraction of other unsaturated oils like soy bean oil and linseed oil which could be improved to within 15 iodine number units using the "segregation process." (Mills, 1952; Switzer, 1952.)

CONCLUSION

This study has shown that it is indeed possible to increase the iodine value of lumbang oil to within 15 iodine number units using urea complexes. This increase shows too that there is a considerable improvement in the drying properties of the oil since the iodine number is a measure of the degree of unsaturation of the sample.

Future application of this method would ultimately depend on the following factors:

1. The industrial benefits that lumbang oil with an increase of 15 iodine number units would give considering the economics of large scale production.

2. The uses for which the raffinate (15 to 20 per cent of the starting oil) could be intended, possibly as foodgrade industrial chemicals.

3. Recovery of urea. The present process could recover the urea crystals to the extent of 96.5 per cent. This could be recrystalized and recycled. The extent of recovery could be subject to improvement so as to cut down losses.

4. Recovery of the solvent—ethanol, ether or chloroform (in place of ether) could be collected, purified and recycled.

ACKNOWLEDGMENT

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I.B. 6.a.) extended to this study and the Philippine Wallboard Corporation (Nasipit Lumber Company, Inc.) for the lumbang oil samples.

BIBLIOGRAPHY

ABU-NASR, A.M., W.M POTTS, and R. HOLMAN (1954) Highly Unsaturated Fatty Acids 11. Fractionation by Urea Inclusion Compounds. Jour. Am. Oil Chem. Soc. 31: 16-20.

BAILEY, ALTON E. (1951). Industrial Oil and Fat Products. New York: Interscience Publishers, Inc, pp. 101 104.

BAILEY, ALTON E. (1951a) Industrial Oil and Fat Products. New York: Interscience Publishers, Inc., p. 175.

BENGEN (1940) Gen. Patent Application O.Z. 12438, March 18. Technical Oil Commission Reel 6, frames 263-270 (in German) and Reel, pp. 143, 135 139 (in English)

EISENHAUER, R.A., and R.E BEAL (1968). Cyclic fatty acids: separation from straight-chain fatty acids by urea adducting Jour. Am. Oil Chem. Soc. 45: 619-621.

HILDITCH, T.P. (1956). The Chemical Constitution of Natural Fats. London: Chapman and Hall, Ltd., pp. 279-341.

MILLS, M.R (1952) An Introduction to Drying Oil Technology New York: Interscience Publishers Inc., pp. 32 46.

SCHLENK, H., and R.T. HOLMAN (1950). The urea complexes of unsaturated fatty acids. Science 112: 19-20.

SWERN D., and W. PARKER (1953). Application of urea complexes in the purification of fatty acids, esters and alcohols. III. Concentrates of natural linoleic and linolenic acids. Jour Am. Oil Chem. Soc. 30: 5-7.

SWITZER, M.K (1952). Continuous Processing of Fats. London: Leonard Hill Limited, pp. 164 185.

ZIMMERCHIED, W.J., R.A DINERSTEIN, A.W. WEITKAMP, and R.F MARSCHNER (1950). Crystalline adducts of urea with linear aliphatic compounds. A new separation process Ind Eng. Chem 42: 1300-1306

ZIMMERCHIED, W.J., R.A., DINERSTEIN, A.W., WEITKAMP, and R.F. MARSCHNER (1961). Memorandum on the Status of Research on Lumbang (Aleurites moluccana) and Baguilumbang (Aleurites trisperma), by the Nasipit Lumber Company, Inc. Philippines, pp. 1 23.

ELECTRONIC AND STRUCTURAL EFFECTS ON RATES AND EQUILIBRIA. VII NUCLEOPHILICITY OF SOME ALIPHATIC AMINO ACIDS *

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Philippine Refining Co, Manila

FIVE TEXT FIGURES

ABSTRACT

Nucleophilic reactivity of some aliphatic amino acids was determined at 190 nm and at 40°C in hexane-water. Dinitrofluorobenzene (DNFB) was used as the reference electrophile. The second order rate constants (Kobs, 10^{-4} liter/mole-sec) by method of least squares are glycine, 0.64; leucine, 3.60; isoleucine, 2.19, valine, 2.17 and alanine, 1.54. Results are explained in the light of current electronic and structural concepts.

We have been conducting studies on the nucleophilic reactivity of amino compounds (Lim *et al*, 1971; Lim *et al*, 1971a; Lim and de Vera, 1970). We observed that some aromatic amines (Lim and de Vera, 1970), alicyclic amines (Lim *et al*, 1971a) and aliphatic amines (Lim *et al*, 1971) do follow simple rules in the relationship of structure with reactivity (Hine, 1962; March, 1968; Sylianco, 1970).

Investigations performed by Sylianco and co-workers on amino acids (Benitez, 1969; Dia, 1971; Laserna, 1967) have prompted us to do quantitative determination of the effect of structure on the nucleophilic reactivity of the amino group in these compounds.

Amino acids comprise one of the most important sets of biological compounds. These have played important roles in metabolism, diseases, and in other areas of medicine (Mahler and Cordes, 1966). Any additional information with regard to their reactivity would be beneficial in the field of physical

* For previous papers in this series, see "Electronic and Structural Effects on Rates and Equilibria. VI Polarographic Reduction of Substituted Benzaldehydes," Philip. Journ. Sc. 102: 79-87.

[1] To whom inquiries should be sent.

biochemistry as well as in molecular biology. For this reason, this study was undertaken.

EXPERIMENTAL PART

Dioxane was obtained as "Baker Analyzed" reagent grade from J.T. Baker Chemical Co., New Jersey, while 2, 4-dinitrofluorobenzene (DNFB) was procured from Aldrich Chemical Co., Wisconsin. Glycine was purchased from Merck and Co., New Jersey, while DL-valine and DL-isoleucine were obtained from Matheson Coleman and Bell, Ohio. DL α-alamine was procured from Hopkins and Williams Ltd., New Jersey and DL-leucine was from General Biochemical Inc., Ohio. All solids whose melting points did not coincide with literature values were recrystallized using standard procedures.

Melting points were determined using a Fischer-Jones Melting Point Apparatus. A Haake Constant Temperature Circulator was used to provide a constant temperature bath for the kinetic runs. All infrared spectral analyses were done on an Infracord Perkin-Elmer Model 137. A Beckman DU Spectrophotometer was used for all UV determinations.

Products of the reaction of amino acids and DNFB were isolated, purified and characterized following the procedures by Levy and Chung, 1955.

To 25 ml of 2.0×10^{-3} M DNFB in dioxane in a 100-ml volumetric flask preheated to 40°C was added same volume of 2.0×10^{-3} M aqueous solution of DL-amino acid which was also preheated to 40°C. The final solution was shaken well while immersed in the constant temperature bath. Aliquots were taken at definite time interval. Absorption at 490 nm using a Beckman DU Spectrophotometer was taken. A 1.0×10^{-2} M DUFB in 50-per cent aqueous dioxane was used as reference. Absorbance values obtained were extrapolated to concentration units with the aid of a prepared reference concentration-absorbance curves of the isolated products. Rate constants were calculated using numerical and least square methods for a second order rate law (Lim *et al*, 1971; Lim *et al*, 1971a; Lim and de Vera, 1970).

RESULTS AND DISCUSSION

At 490 nm only the product was found to absorb (Lim *et al*, 1971; Lim *et al*, 1971a). At the concentration levels used,

the reactants and products were observed to follow Lambert-Beer's Law. Sample curves are shown in Figures 1 and 2.

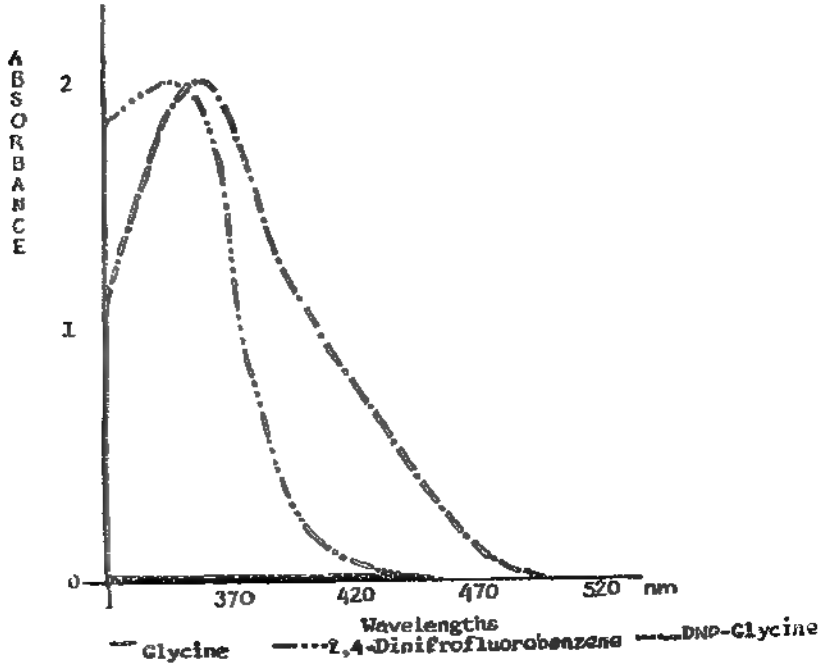


FIG. 1. Absorbance curves of glycine, 2, 4-dinitrofluorobenzene and the product formed in 50-per cent aqueous dioxane.

The rate of reaction was determined by following the rate of formation of the colored product through its absorbance (Lim *et al*, 1971). The second order constants are shown in Table 1.

TABLE 1.—*Second order rate constants for the reaction of amino acids with DNFB in 50-per cent aqueous dioxane at 40°C by method of least squares.*

Amino acid	PKa [1]	R	σ^2	Kobs (× 10⁻⁴ l/mole-sec)
glycine	9.60	H-	—.490	5.64
DL-α-alanine	9.69	CH_3-	0.0	1.54
DL-valine	9.62	$(CH_3)_2CH$-	0.190	2.17
DL-leucine	9.60	$(CH_3)_2CH$-CH_2-	0.125	3.66
DL-iso-leucine	9.68	$(CH^3CH^2)(CH^3)CH$-	.210	2.18

[1] Mahler and Cordes (1966).
[2] Taft (1966).

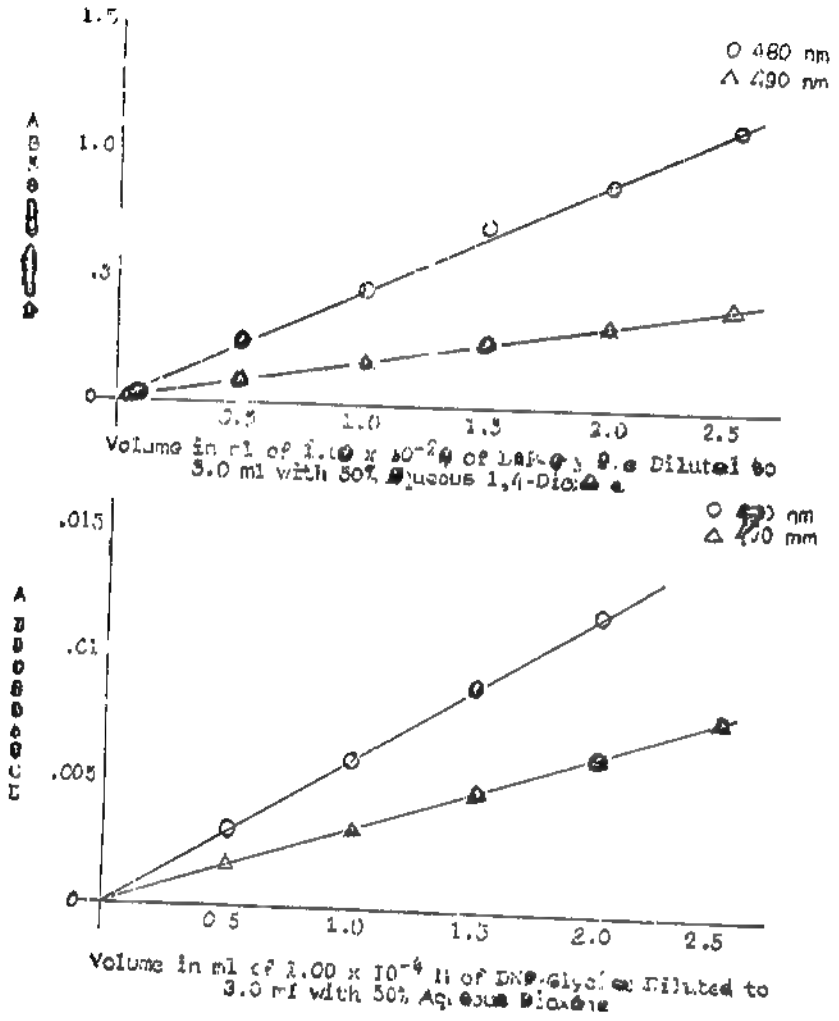


FIG. 2. Absorbance vs. concentration curves of DNP-glycine at 480 nm and 490 nm.

The observed order of reactivity is: glycine>leucine>isoleucine valine alanine.

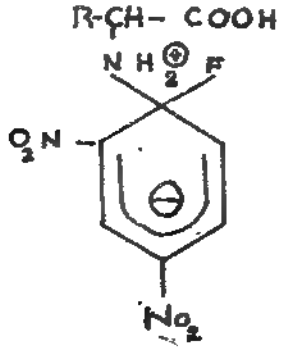
The main difference in the structure of the amino acids used in this study is in the nature of the substituent at the β-position:

$$\begin{array}{l} \beta \quad \alpha \\ R \quad CH\text{—}COOH \\ \quad\quad | \\ \quad\quad NH_2 \end{array}$$

In glycine this β-position is occupied by H; in alanine, methyl; in valine, isopropyl; in leucine, isobutyl and in isoleucine, sec-butyl groups. Since the α-position in all amino acids are the same electronically and sterically any difference in reactivity could not be attributed to this.

The availability of the lone pair of electrons on nitrogen in amino acid is affected by the nature of electron donating alkyl group which occupies the β-position, since this is only one carbon atom away. A stronger electron-donating alkyl group would render the nitrogen more nucleophilic as shown in Table 1; it could be predicted that on the basis of pure inductive effect alone, the nucleophilicity would follow the order: isoleucine>leucine>valine>alanine>glycine. However, this is not observed. In some cases nucleophilicity which is a kinetic parameter goes in the same direction as basicity which is a thermodynamic parameter. From examination of the thermodynamic constants of the amino acids (pKa's) in Table 1, it is evident that the observed sequence does not follow the order due to basicity. If the system studied were controlled by thermodynamic effect alone, the observed rate would be almost the same.

On the basis of steric effect or bulkiness of the alkyl group, the reactivity should follow the order: glycine>alanine>valine >leucine isoleucine. This is just the opposite order compared to that due to inductive effect. It seems that the observed rates could be a composite result of inductive and steric effects. Glycine seems to react the fastest since at its transition state or intermediate (I), there is a negligible steric inhibition as shown:



I

Ia R = H- (glycine)
Ib R = $(CH_3)_2$ CH CH_2- (leucine)
Ic R = CH_3 CH_2 CH (isoleucine)
Id R = $(CH_3)_2$ CH- (valine)
Ie R = CH_3 (alanine)

The reaction of glycine with DNFB relative to the other amino acids is sterically controlled since the hydrogen which occupies the β-position is neither electron-donating nor attracting. Leucine follows glycine in reactivity because it has a four-carbon alkyl group which is quite electron donating. It is less electron-donating than its isomeric case, isoleucine, where the methyl group in the butyl moiety has moved closer to the amino group. The R group of isoleucine is more electron-donating than leucine (Table 1). So from glycine to leucine the difference in reactivity is controlled by inductive effect whereas the reactivity from leucine to isoleucine, the predominating influence is steric effect. There is more steric inhibition of the intermediate (I) in isoleucine than leucine due to higher substitution which occupies the β-carbon in the former. Valine and isoleucine have comparable reactivity, meaning, that the extra steric effect exerted by sec-butyl group at the intermediate is counteracted somewhat by its extra electron donating (by a difference of one carbon atom). Between valine and alanine, the difference is mainly inductive effect rather than steric in nature.

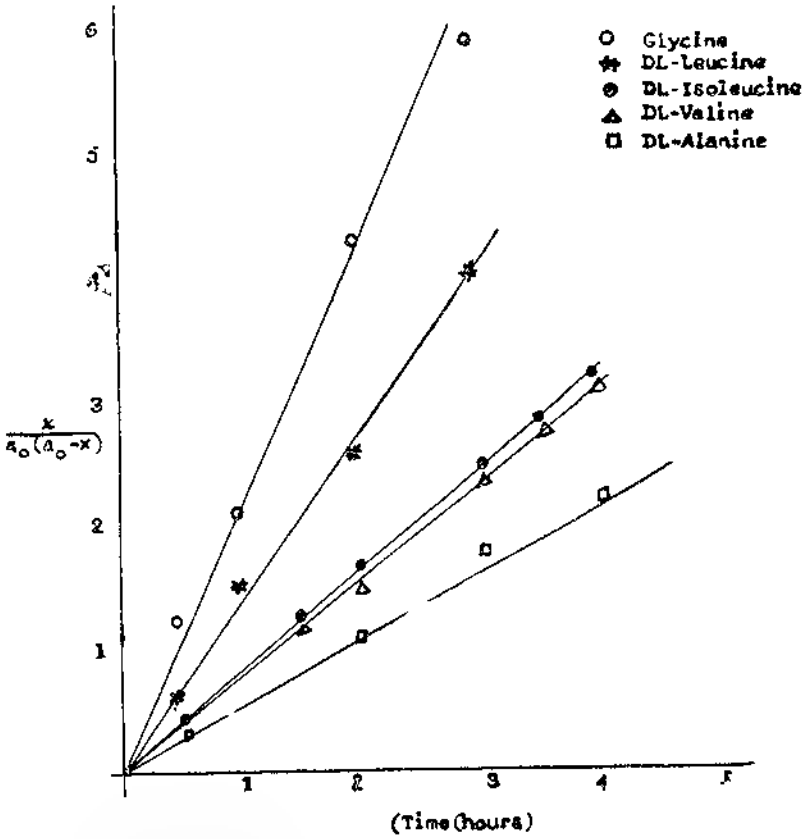


FIG. 3a. Rates of reaction of the amino acids and 2, 4-dinitrofluorobenzene in 50-per cent aqueous dioxane at 40°C.

So it appears that when inductive effect is nil, steric effect becomes the controlling factor. However, three to four carbon-atom alkyl groups can exert inductive influence which can compensate for the steric inhibition at the intermediate. Similar alkyl groups (i.e., butyl) can differ in steric capacity and those which exert less steric influence were found to react faster.

A second order rate law was observed to be followed by the system under study. However, after 4 hours, a leveling-off effect of the monotonic change was evident. Considering all possible factors affecting the reaction we were left with the consideration that the HF generated as the reaction proceeds could be the culprit. This HF being a strong acid becomes

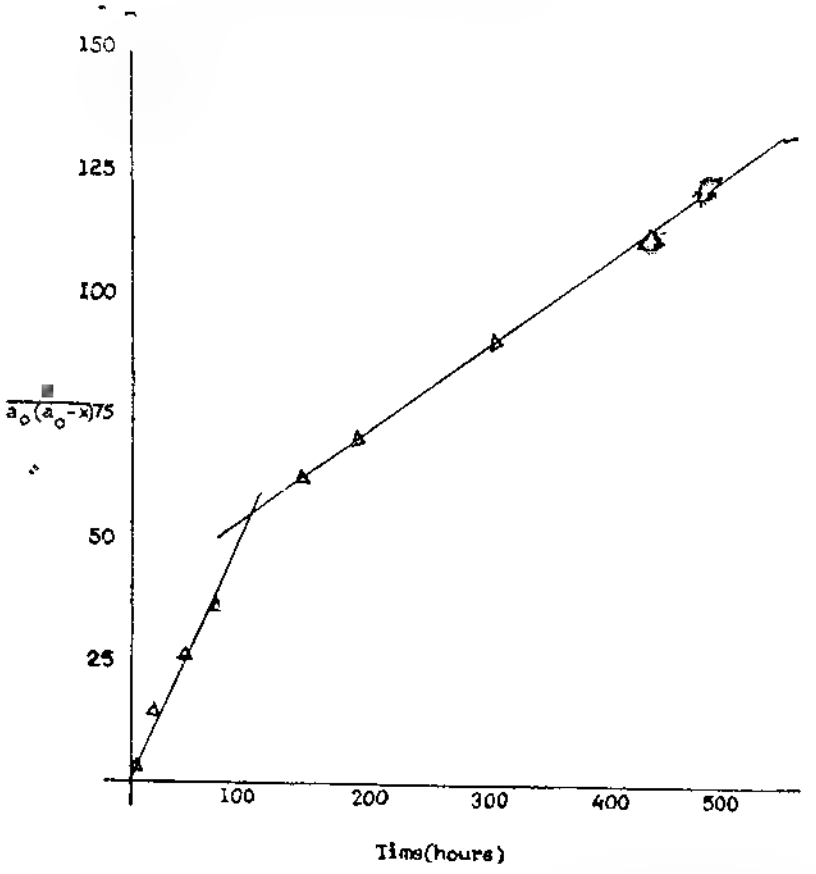


FIG. 3b. Rates of reaction of DL valine and 2, 4 dinitrofluorobenzene indicating change in mechanism in 50 per cent aqueous dioxane at 11°C

significantly high in concentration as the reaction continues and can easily protonate unreacted amino acid To prove this point, a pseudo first order reaction was run where excess amino acid was used to act as an internal buffer system. Indeed the linearity of the rate process was restored (Fig 4).

In conclusion we can safely say that the reactivity of the amino group in some aliphatic amino acids is sensitive to both steric and inductive effects. The second order rate law is complicated by the generated HF from the reaction. The use of excess amino acid can easily buffer the system

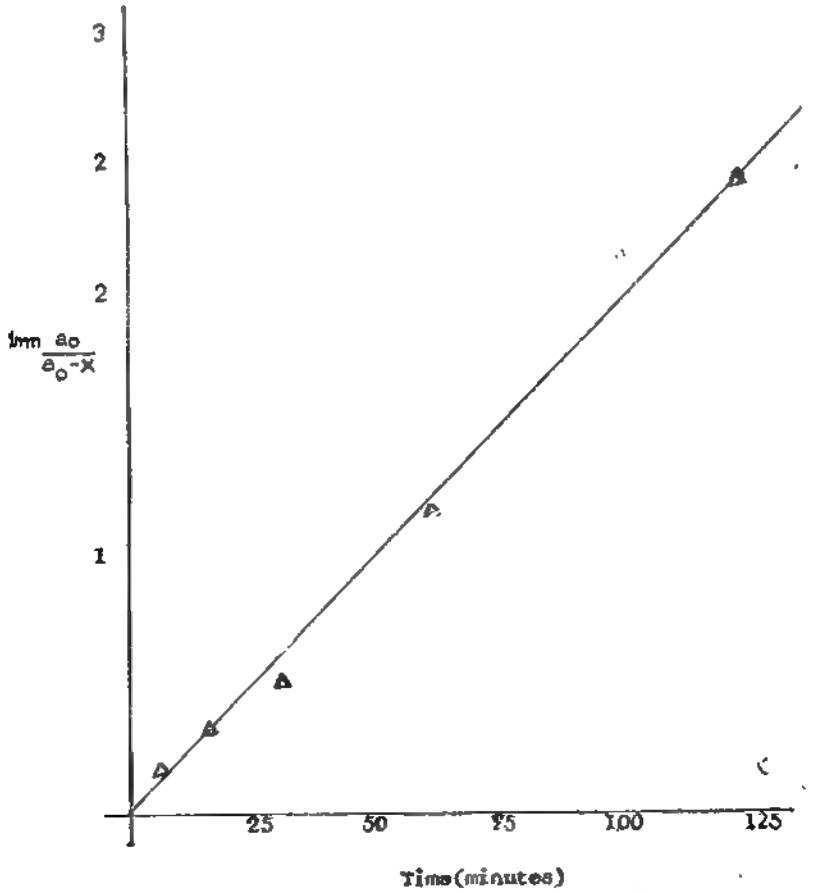


FIG. 4 Pseudo-first order rate of reaction of DL-valine and 2, 4-dinitrofluorobenzene in 50-per cent aqueous dioxane.

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REFERENCES

BENITEZ, LITA (1969). The effect of pH on the Coupling Reactions of Histidine with p-Diazobenzenesulfonic Acid. M.S. Dissertation, Univ. Philip., Diliman, 38 pp.

DIA, VIRGINIA (1971). Non-Kinetic Studies on Hopkins-Cole Reaction. M.S. Dissertation, Univ. Philip., Diliman, 43 pp.

DUMLAO, AMPERFINA (1969). Solvent Effect on the Coupling Reaction of Histidine with p Diazobenzenesulfonic Acid. M.S. Dissertation, Univ. Philip., Diliman, 32 pp.

HINE, JACK (1962). Physical Organic Chemistry. New York: McGraw-Hill Book Co., pp. 100–103

LASERNA, EVELYN (1967). The Coupling Reaction of Histidine and N-methyl Histidine with p Diazobenzenesulfonic Acid. M.S Dissertation, Univ. Philip., Diliman, 30 pp.

LEVY, A.L., and D. CHUNG (1955). A simplified procedure for the synthesis of 2, 4-dinitrophenyl (DNP)-amino acids. Jour. Am Chem. Soc. 77: 2899.

LIM, W.Y., B. LAZARO, and F. MANILIGAS NACINO (1971). Electronic and structural effects on rates and equilibria V. Nucleophilic reactivity of some aliphatic amines Philp. Jour Sci. 101: 261 265.

LIM, W. Y., M. LUGA-RAMIRO, and N DE VERA (1971a). Electronic and structural effects on rates and equilibria, IV. Conformational influence on the nucleophilicity of cyclic and alicyclic amines. Nat. Applied Sci. Bull. 23: in press.

LIM, W Y., and N DE VERA (1970) Polar and steric effects on rates and equilibria I Nucleophilic reactivity of some aromatic amines. Nat. Applied Sc. Bull. 22: 26 37.

MAHLER, H. R., and E. CORDES (1966). Biological Chemistry. New York: Harper and Row, p. 10.

MARCH, J. (1968). Advanced Organic Chemistry. New York: McGraw-Hill Book Co., p. 19.

SYLIANGCO, C. Y. L. (1970). Principles of Organic Chemistry. Quezon City: Aurum Enterprises, pp. 96 100

TAFT, R W. (1966). Steric Effects in Organic Chemistry (ed. by M. S. Newman). New York: John Wiley and Sons, Inc, pp. 590 591.

REACTIONS OF QUINOLINE DERIVATIVES—STUDY OF 2 HYDRAZINO-4-METHYL QUINOLINE

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ONE TEXT FIGURE

ABSTRACT

The reactions of 2 hydrazino 4-methyl quinoline with acetyl acetone, benzoyl acetone, dibenzoyl methane, and ethyl acetoacetate yielded the corresponding 1,2 diazepine (2–4) and 1,3-diazepinone (6). Structures were assigned on the basis of IR and NMR special studies

Reactions of hydrazino derivatives of benzo (f) and benzo (h) quinolines were studied in this laboratory.[1] They were condensed with acetyl acetone and ethyl acetoacetate and cyclised in glycerol (in presence of fused sod. acetate). In literature the aromatic hydrazino compounds have been reported to give triazines and diazepines with nitrous and formic acids. It thus appeared worthwhile to synthesize 1,2–diazepine derivatives of quinoline which itself is important pharmacologically. 1,2-Diazepines have not been studied as exhaustively as the 1,4 and 1,5 diazepines which have been used widely as sedatives, hypnotics, muscle relaxants, and anticonvulsants.

DISCUSSION

2–Hydrazino 4–methyl quinoline 1, and acetyl acetone on refluxing with glicerol in the presence of fused sodium acetate[2] gave a product, 2 ($C_{14}H_{13}N$, 71.4 percent). I.R. spectrum gave a characteristic absorption band at 1605 cm^{-1} thus indicating the presence of 1,2 diazepine moiety in 2.[3] The NMR spectrum (Figure 1) indicated the presence of four aromatic protons at τ 1.95 2.80, 9H of three methyl groups as singlet at τ 7.20, τ 7.35, and τ 7.70 respectively and a proton at nitrogen at τ 0.80. The presence of an olefinic proton at τ 4.0 was the characteristic position for the protons of 1,2-dia-

[1] R.P. Tyagi and B.C. Joshi, Bull. Chem. Soc. (In press.)
[2] Idem.
[3] O. Buchardt *et al*, Acta Chem. Scand. 23: 3125 (1968).

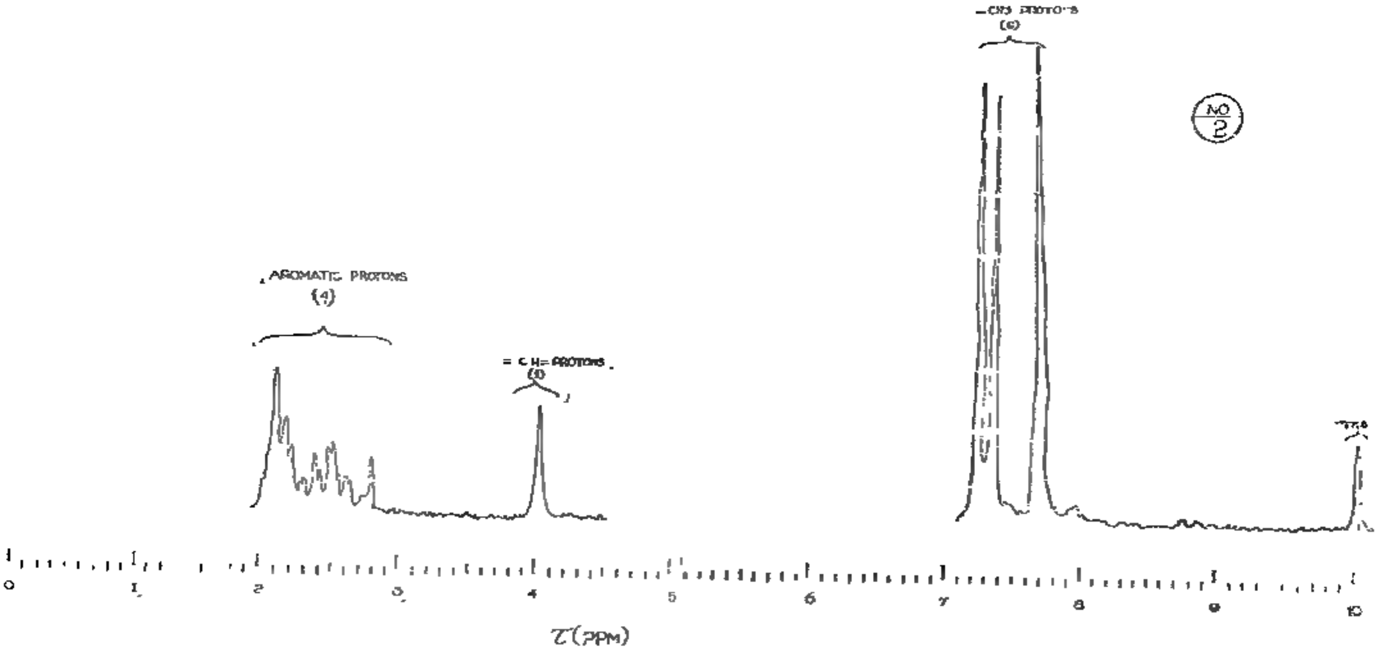
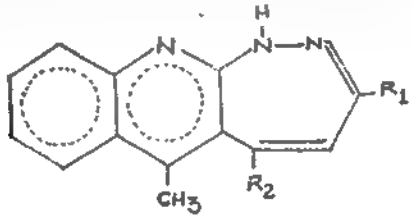


FIG. 1. NMR spectrum of compound 2.

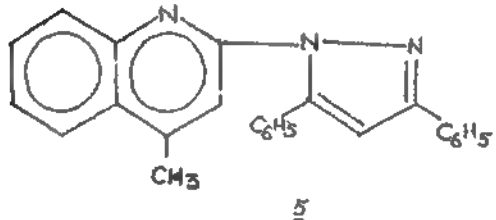
zepine ring. The absence of a proton at position 3 and the presence of a proton at nitrogen and an absorption at 1600 cm^{-1} would account for the fusion of 1,2-diazepine system with quinoline ring and rule out the possibility of a pyrazole ring. As such 2 was assigned the structure as 4-methyl quinoline-(2,3-C)-3,5-dimethyl-1H-1,2-diazepine.



2, - $R_1 = R_2 = CH_3$

3, - $R_1 = C_6H_5$, $R_2 = CH_3$

4, - $R_1 = R_2 = C_6H_5$



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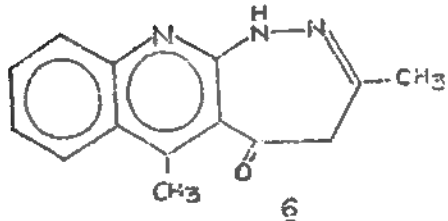

6

Similar observations were made when 1 was reacted with benzoylacetone and dibenzoylmethane and the compounds 3 and 4 were obtained. On similar lines as in 2 compounds 3 as 4-methylquinolino-(2,3-C)-3-phenyl-5-methyl-1H-1,2 diazepine and 4 as 4-methylquinolino(2,3-C)-3,5-diphenyl 1H-1,

2–diazepine were established on the basis of elemental and spectral studies.

Substituting phenyl for methyl groups in 1, 2–diazepine ring brought down the yield of such compounds and a pyrazole derivative 5 (25.3 per cent) obtained was assigned the structure 1 (3,5–diphenyl pyrazolyl-4 methyl quinoline (5) on the basis of elemental and spectral observations.

TABLE 1. *Infrared and nuclear magnetic resonance analyses of synthesized compounds.*

Compound	IR peaks, cm	NMR values										
		NH protons	Number of protons	Aromatic protons	Number of protons	CH protons	Number of protons	CH$_3$ protons	Number of protons	CH$_2$ protons	Number of protons
2	1[illegible]	8	1	1.95–2.80	4	4.8[illegible]	1	7.2[illegible]	3		
								7.3[illegible]	3		
3	1602	[illegible]	1	[illegible]	9	4.9[illegible]	1	[illegible]	3		
								[illegible]0	3		
4	1610	[illegible]	1	2.[illegible]–2.[illegible]	14	3.9[illegible]	1	[illegible]	3		
				2.00–2.40	14			7.4	3		
				3.1–3.3	2						
6	1[illegible]0 1600	1.17	1	2.0–2.80	4			7.40	3	6.90	2
								7.50	3		

TABLE 2. *Details of experimental variable and results.*

No.	Reactants	Time of reflux, hr	Yield	m.p.	Analyses					
					Computed			Found		
					C	H	N	C	H	N
		Hour	*Per cent*	*Degree*						
3	1 + Benzoylacetone + sodium metal + sodium acetate	[illegible].5	[illegible].0	13[illegible]			14.01	[illegible]		14.28
4	1 + Dibenzoylmethane + sodium acetate	4	3[illegible].3	8[illegible]	[illegible]		11.62	[illegible]	[illegible]	11.98
			62.8							
5	1 + Dibenzoylmethane + sodium acetate	4	[illegible].3	1[illegible]			11.63	—		11.48
6	1 + Ethylacetoacetate + sodium metal	1[illegible]	31.8	1[illegible]	70.29	[illegible].43	17.[illegible]1	70.58	6.72	17.82

The reactions of 1 with ethyl acetoacetate yielded a crude mixture (74 per cent) from which only a compound 6 ($C_{13}H_{14}N_2O$, 31.8 per cent) could be separated in pure form. Infrared spectrum of 6 showed absorption bands at 1700 and 1600 cm^{-1} indicating the possibility of the presence of a diazepinone ring. The NMR spectrum indicated the presence of four aromatic protons at τ 2.8 1.95, 6H as singlet of two methyl groups at τ 7.40 and τ 7.50, 2H of methylene group as singlet at τ 6.90

and a proton at nitrogen at τ 1.15. The proton at position 3 was also found to be absent. On the basis of spectral observations **6** was therefore, assigned the structure as 4-methylquinolino-(2,3-C)-3-methyl-4,5-dihydro-1H-1,2-diazepin-5-one.

EXPERIMENTAL

Melting points are uncorrected. Micro analyses and NMR spectra were mostly done at CDRI, Lucknow. NMR spectra were determined on Varian Model A-60 in $CDCl_3$ using TMS as internal indicator. Chemical shifts are listed as τ values (ppm).

Synthesis of 4-methylquinolino-(2,3,-C)+3.5-dimethyl-1H-1,2-diazepine (2).—2-Hydrazinolepidine (3.46., 0.02 M) and anhydrous sodium acetate (3.0 g) were taken in glycerol (30 ml). The reaction mixture was stirred for a while at 25 to 30°. Acetyl acetone[1] (2.0 g, 0.02 M) was added drowse over a period of 0.5 hr and the reaction mixture was heated to 150° to 200°. It was further refluxed for 0.75 hr and the reaction mixture was poured over crushed ice. The crude product was crystallized from alcohol, m.p. 107° (yield 71.4 per cent).

Analysis: Calcd. for $C_{15}H_{15}N_3$:
C, 75.94 per cent; H, 6.32 per cent; N, 17.72 per cent.
Found: C, 75.72 per cent; H, 6.20 per cent; N, 17.69 per cent.

ACKNOWLEDGMENT

The authors are thankful to the Head of the Chemistry Department for providing the necessary facilities and to C.S.I.R. authorities for providing junior research fellowship to the senior author.

[1] When the diketones are solid, the alcoholic solution was used in the reaction.

STEPWISE FORMATION AND THERMODYNAMICAL PARAMETERS OF THORIUM COMPLEXES WITH SALICYLALDOXIME

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[Received ...]

ABSTRACT

Thorium (IV) complexes with salicylaldoxime (H_2BA) have been studied by established potentiometric techniques. The thermodynamical formation constants have been calculated by extrapolating the measured formation constants at various ionic strengths to zero ionic strength. The stepwise protonation constants of the diprotonic ligand and stability constants of thorium chelates have been determined. The step thermodynamical parameters enthalpy, entropy changes and standard free energy of thorium chelates have been determined and correlated. Some light has also been thrown on the structure of complex chelate.

INTRODUCTION

Brady,[1,2] at first, suggested the use of salicylaldoxime (H_2BA), as a chromogenic ligand, by preparing complexes with cobalt and nickel. Later on Bontelsky and Jungries[3] isolated and studied its copper complex, who also studied further the complexes of cobalt and nickel. The literature has reported the complexes of barium, strontium and calcium, magnesium and beryllium[4,5] along with other metals.[6-9]

Present work deals with stepwise formation of thorium chelates with salicylaldoxime (H_2BA). For this Bjerrum-Cal-

[1] O. L. Brady and M. M. Myers, J. Chem. Soc. 1599 (1930).
[2] O. L. Brady, J. Chem. Soc. 105 (1931).
[3] M. Bontelsky and E. Jungries, J. anorg. allgem. Chem. 3: 38 (1956).
[4] P. O. Lumme, Suomen Kem. 309: 194 (1957), 313: 283 (1959).
Z. Holzbecher, Collection Czech. Chem. Commun. 24: 3117 (1959).
R. Ripan, G. Kiss, and Z. Szekely, Acad. Rep. Populare Romine Filiala Cluj, Studii Cercetari Chim. 11: 259 (1960).
K. L. Tabaljwala, K. V. Venkatachalam, and M. B. Kabadi, J. inorg. nucl. Chem. 26: 1011 (1964).
[5] M. S. Kachhawaha and A. K. Bhattacharya, Z. anorg. allg. Chem. 325: 321 (1963).
[6] K. Burger and I. Egyred, J. inorg. nucl. Chem. 27: 179, 2361 (1965).

vin pH titration technique[10,11] was adopted at different ionic strengths and temperatures in 1:1 water-dioxan medium. Thorium forms 1:1 chelates in distinctly acid medium at pH 3.25, another 1:2 chelate in alkaline range at pH 7.25, but both the chelates are of yellow color. The possibility of the formation of polynuclear complexes is ruled out owing to the fact that the results obtained at different concentrations of thorium and H_2BA are the same. The study includes the determination of proton-ligand and metal-ligand stability constants at different ionic strengths and at different temperatures. The step thermedynamical stability constants have been calculated by extrapolating to zero ionic strength, and the corresponding free energy changes have, also, been calculated. Other step thermodynamical parameters like enthalpy and entropy have also been evaluated.

EXPERIMENTAL

Chemicals: Thorium nitrate, potassium nitrate, dioxan, salicylaldoxime, nitric acid, oxalic acid, and sodium hydroxide (ANALAR B.D.H., grade): phenolphthalein (B.D.H., grade).

Standard thorium nitrate, potassium nitrate, oxalic acid, and salicylaldoxime solutions were prepared by dissolving exactly weighed amounts in 1:1 double-distilled-dioxan mixture.

Sodium hydroxide solution was prepared and standardized against standard oxalic acid solution using phenolphthalein solution as indicator in 1:1 water-dioxan mixture. Standard nitric acid solution was prepared by diluting it with 1:1 water-dioxan mixture and standardized by titrating against standard sodium hydroxide solution.

Apparatus: Standard pipettes and burettes, beakers (PYREX glass) of capacity 50 and 100 ml were used. Leeds and Northrup pH-meter operated at 250 v/50 cycles A.C. mains and an electrically regulated thermostat with an accuracy of $\pm$ 0.1° were used.

Bjerrum-Calvin potentiometric technique was adopted where:

(a) 5 ml of 0.1M HNO_3 and known volume of standard potassium nitrate solution,
(b) 25 ml of 0.02M salicylaldoxime solution and mixture (a),
(c) 5 ml of 0.005M thorium nitrate solution and mixture (b),

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[10] J. Bjerrum, Metal amine formation in aqueous solution, Haase, Copenhagen (1941).

[11] M. Calvin and K. W. Wilson, J. Am. Chem. Soc. 67: 2003 (1945).

were taken in different beakers and in each case of these three titration, against standard sodium hydroxide solution (005M), the total volume was maintained at 50 ml before titrations.

The three titration curves (i) acid titration curve, (ii) ligand titration curve, and (iii) the chelate titration curve, were obtained by plotting pH against the volume of the sodium hydroxide solution added. The shape of these curves was the same as that obtained by Al-Komser and Sen.[12]

Accurate values of the stability constants of th(IV)–H_2BA system chelates have been obtained as no electrode corrections were required; and the protonation constant of the ligand in such a medium serves a good intermediate for calculating the stability constants of these chelates. All experiments were carried out in nitrogen atmosphere.

Thorium forms 1:1 chelate in a distinctly acid medium at pH 3.25, and another 1:2 chelate is formed in the alkaline range at pH 7 23 with salicylaldoxime. It has been observed that the formation curves remain incomplete at lower ionic strengths. Since the curves are symmetrical at $\bar{n}_A$1.0, so a light yellow color appears from colorless in the pH range from 7.60 to 8.60 at all ionic strengths. Further, as pH corresponds to $\bar{n}_A$ value 1.5, at different ionic strengths, it suggests that the transition from colorless H BA to light yellow HBA specie takes place in solution.

CALCULATIONS

Proton-ligand stability constants.—The association of protons (H) with the ligand (I.) shorn of all dissociable protons is

$$L + H \rightleftharpoons LH$$

$$LH + H \rightleftharpoons LH_2, \ldots\ldots \text{etc.}$$

The average number of protons ($\bar{n}_A$) associated with the ligand molecule is defined as

$$\bar{n}_A = \frac{\text{total concentration of protons bound to ligand}}{\text{total concentration of ligand not bound to metal}}$$

[12] K.M.J. Al-Komser and B. Sen, Inorg. Chem. 2: 1219 (1963).

Thus, for a ligand with two dissociable protons,

$$\bar{n}_A = (C_{LH} + 2C_{LH_2})/(C_L + C_{LH} + C_{LH_2}) \quad \ldots\ldots\ldots (1)$$

$$\text{or} \quad \bar{n}_A = (K_1^H C_1^H + 2K_1^H K_2^H C_H 2)/(1 + K_1^H C_H + K_1^H K_2^H C_H 2) \quad \ldots\ldots (2)$$

where K_1^H and K_2^H are the first and second ligand stability constants, and C represents the concentration. Rearrangement of equ'n (2) gives,

$$\bar{n}_A = (\bar{n}_A - 1)C_H + K_1^H - K_1^H K_2^H (2 - \bar{n}_A) C_H / (\bar{n}_A - 1) \quad \ldots (3)$$

The value of protonation constant can be determined by substituting different values of $\bar{n}_A$ and corresponding values of pH in equ'n (3) and then taking the average value.

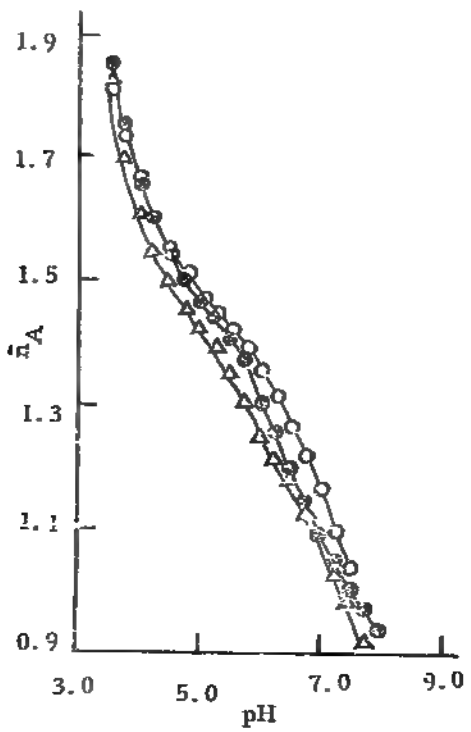


FIG. 1. Proton-ligand formation curves of H_2BA at 30°C.

●-● $\mu = 0.02$
△-△ $\mu = 0.06$
○-○ $\mu = 0.10$

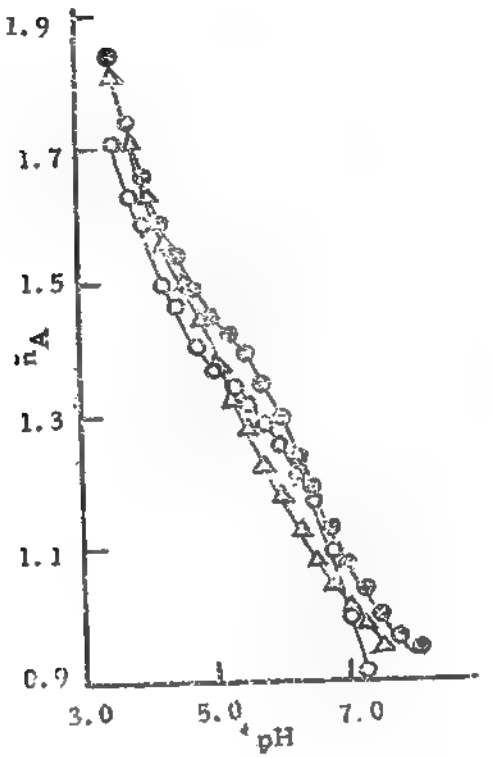


FIG. 2. Proton-ligand formation curves of H_2BA at 40°C.

●-● μ = 0.02
▲-▲ μ = 0.06
o-o μ = 0.10

In order to calculate the value of protonation constant by other methods, Figs. 1 to 3 represent the formation curves. Suitable computation methods were applied to determine the values of protonation constants. For the determination of $\bar{n}_A$ values the following equ'n (4) was used:

$$\bar{n}_A = yTC_L^\circ + (V' - V'') (N^\circ + E^\circ)/(V^\circ + v')/TC_L^\circ \quad \ldots (4)$$

where v' and v'' stand for the volumes of the alkali required in the acid (curve) and ligand (curve) titrations respectively, at a particular pH value, N° is the normality of sodium hydrox-

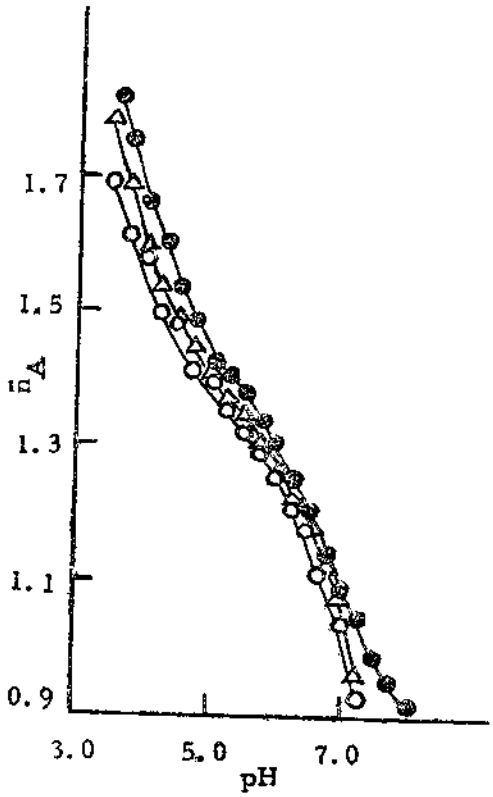


FIG. 3. Proton-ligand formation curves of H_2BA at 50°C.

o-● μ = 0.02
Δ-Δ μ = 0.06
o-o μ = 0.10

ide solution: E° and TC°_L are the overall concentrations of the acid and the ligand in the titration mixtures, V° is the total volume of the mixture and y is the number of the dissociable protons.

Metal-ligand stability constants. The stepwise complex formation between a metal ion M and N, and ligand L shorn of all ionizable H-ion, the equilibria can be represented by

$$ML_{n-1} + L \rightleftharpoons ML_n \quad (n = 1, 2, 3, \ldots\ldots N)$$

The stepwise formation constants are given by

$$K_n = C_{ML_n} / C_{ML_{n-1}} \quad (n = 1, 2, 3, \ldots\ldots\ldots N)$$

where K_n is the *nth* metal-ligand stability constant.

The formation constants are obtained from the analysis of the formation curves (Figs. 4–6) drawn between ñ and PL. The computation methods of Irving and Rosotti[13] and Sen[14] were used. The value of ñ, the average number of ligands attached per metal ion present in whatever form is given by the following equ'n (5),

$$\bar{n} = (v'' - v') [N^\circ + E^\circ + TC_i^\circ (y - \bar{n}_A)] / (v'' + V^\circ) \bar{n}_A TC_M^\circ \quad \ldots (5)$$

where TC_M° represent the total concentration of the metal present in the solution, v" and V" ' denote the volumes of the alkali required in the case of ligand and complex titrations respectively at the same pH. Equ'n (5) can be simplified with the help of equ'n (4),

$$\bar{n} = (v''' - v'') (N^\circ + E^\circ) / (V^\circ + v') \bar{n}_A TC_M^\circ \quad \ldots\ldots\ldots (6)$$

ñ values were generally determined with equ'n (6).

[13] H. Irving and H.S. Rossotti, J. Chem. Soc. 3397 (1953); 2904 (1954).
[14] B. Sen, Anal. Chem Acta 27: 515 (1962).

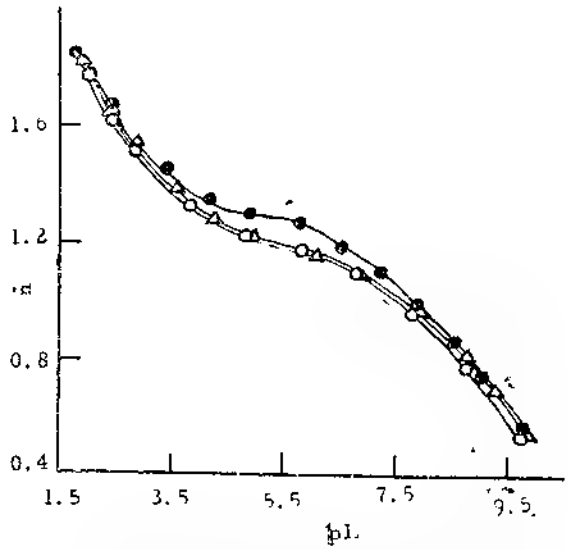


FIG. 4. Metal-ligand formation curves: Tl. -H_2BA at 30°C.

o-● μ = 0.02
Δ-Δ μ = 0.06
o-o μ = 0.10

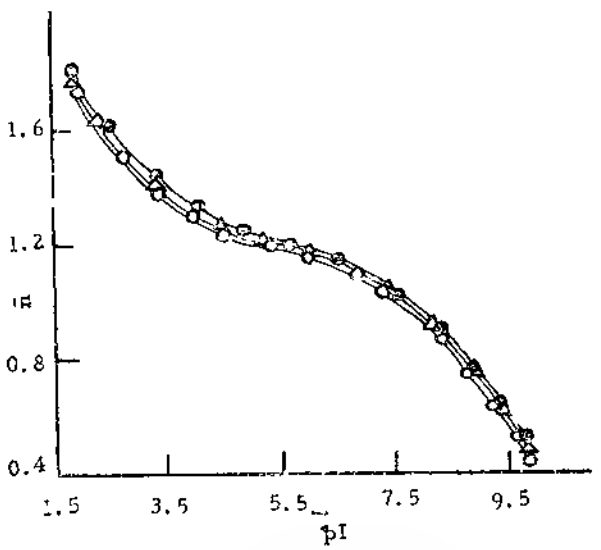


FIG. 5. Metal-ligand formation curves: Th—H_2BA at 40°C.

o-● μ = 0.02
Δ-Δ μ = 0.06
o-o μ = 0.10

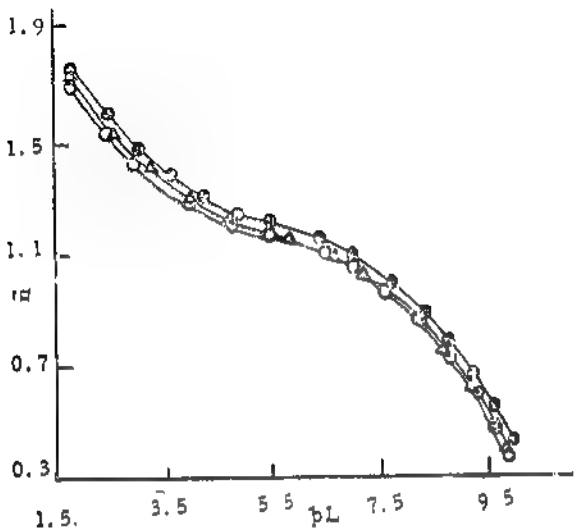


FIG. 6. Metal-ligand formation curves: Th—H_2BA at 50°C.

⊕-⊕ $\mu = 0.02$
Δ-Δ $\mu = 0.06$
○-○ $\mu = 0.10$

The free ligand exponent (PL) was calculated from

$$pL = \log_{10}\left[\frac{\sum_{n=0}^{n=j} \beta_n^H \,(\text{antilog } B)^{-n}}{(TC_L^0 - nTC_M^0)} \cdot \frac{V^0 + v''^{n}}{V^0}\right] \quad \ldots\ (7)$$

where β_n^H is the overall proton-ligand stability constant and B is the pH of the solution. The term $(V^0 + v'')/V^0$ is the volume correction term—which is necessary for accuracy.

RESULTS

Proton-ligand system.—The formation curves in Figs. 1–3 appear to be, almost, straight lines which have been extended between 0 and 2 on the $\bar{n}_A$ scale. The dissociation of the ligand takes place in the following two steps:

$$H_2\,BA \rightleftharpoons HBA^- + H^+$$

$$HBA^- \rightleftharpoons BA^{-2} + H^+$$

The values of proton-ligand stability constants at ionic strengths 0.02, 0.06 and 0.10 and at temperatures 30°, 40°, and 50°C are given in Table 1.

TABLE 1.—*Protonation constants of salicylaldoxime.*

Temperature °C	Methods	n	log K_1	log K_2	log β_n^H
30°	Half nA values	0.02		4.75	
	Midpoint slope mean	0.02	10.35	4.75	15.10
		0.02	10.35	4.75	
	Half nA values	0.06		4.50	
	Midpoint slope mean	0.06	10.10	4.50	14.60
		0.06	10.10	4.50	
	Half nA values	0.10		4.25	
	Midpoint slope mean	0.10	9.90	4.25	14.15
		0.10	9.90	4.25	
40°	Half nA values	0.02		4.70	
	Midpoint slope mean	0.02	10.20	4.70	14.90
		0.02	10.20	4.70	
	Half nA values	0.06		4.45	
	Midpoint slope mean	0.06	9.95	4.45	14.40
		0.06	9.95	4.45	
	Half nA values	0.10		4.20	
	Midpoint slope mean	0.10	9.80	4.20	14.00
		0.10	9.80	4.20	
50°	Half nA values	0.02		4.65	
	Midpoint slope mean	0.02	10.15	4.65	14.80
	Half nA values	0.02	10.15	4.65	
	Midpoint slope mean	0.06		4.40	
		0.06	9.90	4.40	14.30
	Half nA values	0.06	9.90	4.40	
		0.10		4.20	
	Midpoint slope mean	0.10	9.75	4.15	13.90
		0.10	9.75	4.10	

Metal-ligand system.—Figs. 4–6 show the formation curves which are of particular shape. The stability constants were read-off from (i) graph or were calculated by other computational methods, (ii) midpoint slope, (iii) corrections term, and (iv) average value methods. Table 2 shows the mean values of stability constants of the metal chelates by different methods.

STEP THERMODYNAMICAL PARAMETERS

The thermodynamical stability constants were obtained by extrapolating the measured formation constants to zero ionic strengths (Figs. 7 and 9), and the ligational standard free

TABLE 2.—*Stability constants of Th(IV)-H₂BA system.*

log K_n	30°C	40°C	50°C	μ
log K_1	10.00	9.65	9.70	0.02
log K_2	3.10	3.00	2.95	0.02
log b_2	13.10	12.65	12.65	0.02
log K_1	9.90	9.75	9.60	0.06
log K_2	3.00	2.85	2.75	0.06
log K_3	12.90	12.60	12.35	0.06
log K_1	9.80	9.70	9.50	0.10
log K_2	2.90	2.70	2.60	0.10
log K_3	12.70	12.40	12.10	0.10

energy was also calculated from

$$\Delta F^\circ = 2.303\ RT\ \log K^{\mu=0} \qquad \ldots\ldots\ldots\ldots\ (8)$$

where log $K^{\mu=0}$ is the extrapolated value of the formation at zero ionic strength.

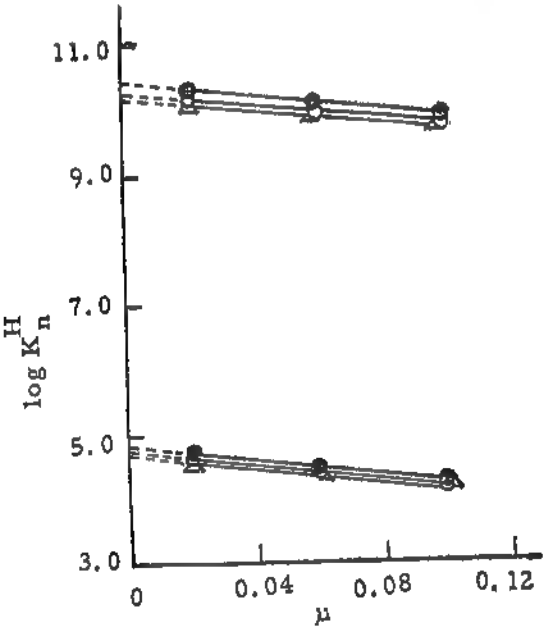


FIG. 7. Log K_n^H vs. μ—H—H_2BA system

●-● 30°C
o-o 40°C
Δ-Δ 50°C

The step thermodynamical formation constants have been obtained by extrapolating the value of logarithms of step formation constants at various ionic strengths to zero ionic

strength. Their sum has been symbolized as calcd. log K $_{\mu=0}$ The exptl. log K $_{\mu=0}$ was obtained by extrapolating the values of overall concentration formation constants β_n^H or K_c at various ionic strengths to zero ionic strength (Figs. 8 and 10.).

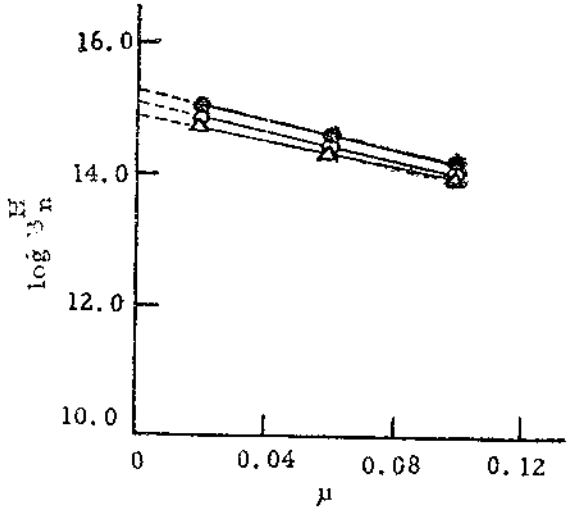


FIG. 8. Log β_n^H vs. μ—H—H_2BA system.

●-● 30°C
○-○ 40°C
△-△ 50°C

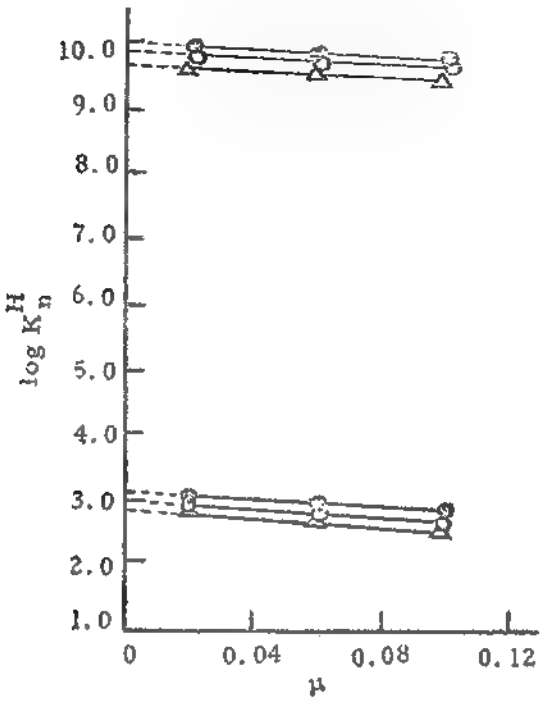


FIG. 9. Log. K_H vs. μ—Th—H_3BA system

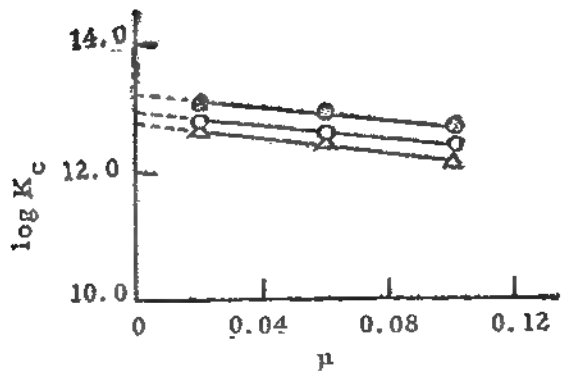


FIG. 10. Log K_c vs. μ—Th—H_3BA system

●-● 30°C
○-○ 40°C
Δ-Δ 50°C

TABLE 3.—*Thermodynamical stability constants and ligational standard free energy.*

Parameters	H_4	Th(IV)	Temp., °C
$\log K_1^{\mu=0}$	10.15	10.07	
$\log K_2^{\mu=0}$	4.85	3.15	
Calcd. $\log K^{\mu=0}$	15.30	13.20	
Expt. $\log K^{\mu=0}$	17.53	15.20	
Δ F°, K cal/mole	11.53	13.03	30
Δ F°, K cal/mole	6.53	4.37	
Calcd Δ F°, K cal/mole	−24.82	18.20	
Expt. Δ F°, K cal/mole	21.22	−18.20	
$\log K_1^{\mu=0}$	10.50	9.90	
$\log K_2^{\mu=0}$	4.80	3.05	
Calcd. $\log K^{\mu=0}$	17.10	12.95	
Exptl. $\log K^{\mu=0}$	17.10	12.95	
Δ F°, K cal/mole	11.65	11.18	40
Δ F°, K cal/mole	7.65	4.33	
Calcd Δ F° K cal/mole	21.76	15.55	
Exptl Δ F°, K cal/mole	21.73	−15.55	
$\log K_1^{\mu=0}$	10.90	9.85	
$\log K_2^{\mu=0}$	4.70	3.00	
Calcd. $\log K^{\mu=0}$	11.90	12.85	
Exptl. $\log K^{\mu=0}$	11.90	12.85	
Δ F°, K cal/mole	−15.08	14.68	50
Δ F° K cal/mole	−7.00	4.44	
Calcd. Δ F°, K cal/mole	22.08	−19.00	
Exptl Δ F° K cal/mole	22.08	−19.00	

The values of the step thermodynamical stability constants and ligational standard free energy have been given in Table 3.

Enthalpy or the changes in heat content, H was determined by either using van' Noff isochore,

$$\frac{d \ln K}{dt} = \frac{\Delta H}{RT^2} \qquad \ldots\ldots\ldots\ldots\ldots\ldots \quad (9)$$

or direct thermal measurements using calorimetry.

Integration of equ'n (9) gives,

$$\log \frac{K_2}{K_1} = \frac{\Delta H}{R} \cdot \frac{T_1 - T_2}{T_1 T_2} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (10)$$

Equation (9) was utilized for calculating ΔH.

Entropy was calculated by

$$\Delta F = \Delta H - T\Delta S \qquad \ldots\ldots\ldots\ldots\ldots\ldots (11)$$

The values of enthalpy and entropy changes have been given in Tables 4 and 5.

TABLE 4—*Changes in enthalpy of Th(IV) -H-BA system.*

Parameters	H+	Th(IV)
ΔH°_1, K cal mole	6.65	−6.19
ΔH°_2, K cal mole	−3.33	−3.29
Calcd ΔH°, K cal /mole	−9.98	−9.48
Exptl ΔH°, K cal mole	10.1	−9.51

TABLE 5.—*Changes in the entropy of Th(IV) -H- BA system.*

Parameters	H+	Th(IV)
ΔS°_1, e.u.	23.91	21.39
ΔS°_2, e.u.	9.36	2.3
Calcd ΔS° e.u	33.27	23.71
Exptl ΔS°, e.u.	20.46	23.96

DISCUSSION

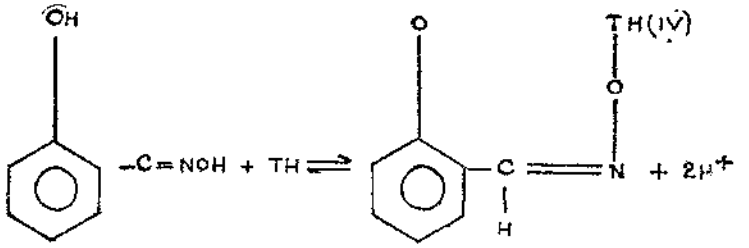
Figs. 1 3 represent the formation curves of the ligand at various ionic strengths and temperatures. The formation curves are almost straight lines which are extended on the $\bar{n}_A$ scale from 0 to 2 indicating, thereby, the dissociation of H_2BA. Protonation constants at either read-off directly from (i) the curve where $\bar{n}_A$ is plotted against pH, or calculated by (ii) midpoint slope method. A perusal of the curves show that they remain incomplete and become prominent as ionic strength decreases. As the curves appear symmetrical at $\bar{n}_A \approx 1.0$, the color changes from colorless to light yellow at all ionic strengths in the pH ranging from 7.60 to 8.60—which cor-

responds to $\bar{n}_A$ value 1.5 at different ionic strength suggests the transition from colorless H_2BA species to light yellow HBA^- species in solution.

It has been observed that first proton of H_2BA was liberated at pH 2.80 and the second proton was liberated at pH 6.5. At first the complex between thorium and H_2BA was formed in the ratio of 1:1 at pH 3.25 and then in the ratio of 1:2 at pH 7.25. From Table 3 it appears that the value of $\log K_1^{\mu\ 0}$, in the case of 1:1 chelate, of H^+ and Th(IV) is approximately of the same order but the value of $\log K_2^{\mu\ 0}$ of Th(IV) is less than that of H^+, in the case of 1:2 chelate. It is further, apparent from Table 3 that the calculated and the experimental values of the ligational standard free energies are in good concordance.

From Table 5 it is apparent that value of $\triangle S_1^\circ$ of the metal chelate is approximately of the same order as that H^+, but the magnitude of $\triangle S_2^\circ$ is smaller in the case of thorium chelate in comparison to the protonated complex. The low value of entropy signifies that Th(IV) chelate is less stable.

The review of Mellor[15] throws some light on the structure of thorium chelates. In his review it is emphasized that six-membered ring structure has been confirmed by analysis for Ni(II), Pd(II) and Pt(IV) chelates of H_2BA, which are planar and stable. In the present case the reaction between thorium and H_2BA



leads to the formation of a seven-membered ring, which is less stable than a six-membered ring. Further, lower entropy value supports the seven-membered ring in the thorium chelate with salicylaldoxime. Hence it may be concluded that thorium chelate has seven-membered ring.

[15] D. P. Mellor, Chem. Rev. 33: 137 (1943).

EFFECT OF GAMMA RADIATION AND PACKING ON THE POSTHARVEST LIFE OF GUAVA (PSIDIUM GUAJAVA L.)

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TWO TEXT FIGURES

ABSTRACT

Guava fruits were subjected to gamma irradiation and packed in polyethylene film or wax paper to observe and compare their taste and effect on shelf life, weight loss during storage, changes in pectin constituents, ascorbic acid, total soluble solids, and acidity.

Optimum radiation dose at 30 Krad delayed ripening of guavas by 7 days while those packed in wax paper polyethylene film were delayed 6 and 7 days, respectively. Four microorganisms isolated from decaying guavas were noted.

INTRODUCTION

Guava is a very delicious and nutritious fruit of Punjab (Central region of Pakistan) and is liked by people of all walks of life. It is one of the riches sources of pectin and vitamin C, and contains appreciable quantities of vitamin A, vitamin B complex, iron, phosphorus and calcium (Munsell *et al*, 1953; Navia *et al*, 1955; Chughtai and Khan, 1960) Guava fruit is known as the apple of the tropics in many countries. The commercial use of this fruit is for jelly making. Guava marmalade, chateneys, cheese, butter, paste and gumdrops are other products from guava consumed by the public.

Guava, being a perishable commodity cannot be sent to distant markets for consumption, hence there is a good deal of wastage in areas of abundance. Ionizing radiation and packing treatments have been successfully used for the extension of shelf life of many fruits (Sattar *et al*, 1970; Scott and Roberts, 1966, 1968). However, very little work has been done on the extension of storage life of this fruit. Mathur (1963) reported delayed ripening of guavas at 30 Krad dose level. He also found a marked reduction in the decay of this fruit due to irradiation. Farooqi *et al*, (1967), while working on green commercially ripe guavas, reported delay in senescense in both types of fruits and the optimum dose suggested was 30 Krad.

One of the undesirable side-effects of irradiation treatment is softening of tissues (Glegg *et al,* 1956; Smock and Sparrow, 1957) and Maxie and Sommer (1964) considered it the most important limiting factor in the application of radiation to fruits and vegetables. Doses above 25 Krad cause tissue degradation which may be correlated with radiation induced changes in pectin and cellulose (Kertesy *et al,* 1964). No study of changes occurring in the pectin contents of guavas subjected to postirradiation storage has been reported previously.

The present investigation was undertaken to study the effect of gamma radiation and packing treatments on the postharvest life, physico-chemical characteristics and sensory evaluation of guava fruit.

MATERIALS AND METHODS

Mature green guavas of *Safeda* variety were harvested from an orchard about 5 miles from the laboratory. Fruits of almost equal size and free from bruises were selected and randomly distributed into equal lots for radiation and packing treatments. Eight to ten fruits were held in polyethylene bags and subjected to gamma radiation doses of 0, 10, 20, 30, 40, 50, and 75 Krad. Radiation was carried out in the "Gamma Cell 220" Co^{60} source (Atomic Energy of Canada Ltd.). Dose rate at the time of radiation was 4 Krad/min. as determined by $FeSO_4$ dosimetery (Aziz and Dyne, 1963). Irradiated and unirradiated lots of guavas were kept in perforated wooden boxes lined with newspaper. To study the effect of packing material, two lots of unirradiated guavas were held separately in wooden boxes lined with either wax paper or polyethylene film.

All boxes containing experimental fruit were stored at room temperature (25°± 2°C).

Weight loss was recorded from a unit of 10 fruits kept separately from the start of the experiment. Visual observations of the ripening process were recorded daily. Consumer acceptability was determined by the use of a nine-point hedonic scale (Larmond, 1970).

Four randomly drawn fruits from each treatment were cut into pieces (excluding seeds) and homogenized in a Waring blendor. Duplicate samples were taken from this pool for biochemical analyses. Acidity, total soluble solids (TSS) and ascorbic acid were determined by the method of AOAC. Total

soluble solid-acid ratio was also calculated. Different pectin fractions were determined by the method of Rouse and Atkins (1955). The results were statistically treated using analyses of variance (Cochran and Cox, 1965).

Microorganisms responsible for the decay of guavas were determined by transferring samples from spoiled parts onto malt extract agar having a pH of 5.0. The agar plates were incubated at 30°C for 2 days. Colonies of fungi that had developed were isolated and subcultured on the same medium and identified by the slide culture technique.

RESULTS AND DISCUSSION

Visual observations.—There was no direct or immediate effect of irradiation on the sensory characteristics of guavas except that there was slight softening beyond 50 Krad dose levels. On the second day of storage, ripening started in the control and 10 Krad-treated samples and they were completely ripe on the 4th and 5th days, respectively. Packed and 20 to 40 Krad-treated samples were still hard and green on the second day, but yellow spotting and softening developed in the 50 Krad and above irradiated samples. Guavas packed in polyethylene film, wax paper and irradiated at 30 to 40 Krad ripened after 11, 10 and 9 days, respectively. Twenty Krad-treated fruits were slightly over-ripe, while all other samples were over-ripe, spotted, shrivelled and decayed on the 9th day.

It is clear from these results that optimum radiation dose for causing delay in ripening of guavas is 30 Krad. Similar results were obtained by Mathur (1963) and Farooqi *et al* (1967). Packing in polyethylene film also extended the shelf life for about a week. Australian workers (Scott and Roberts 1966, 1968) also found polyethylene film useful in extending the shelf life of bananas. Due to the enclosure, exchange of gases was limited, thus the atmosphere around the fruit was modified as evidenced by packs of water droplets that appeared on the fruit surface and inside the polyethylene bag. This encouraged microbial growth but probably could be overcome by the use of some moisture absorbent in the pack and a fungicide on the surface of the fruit.

Weight loss.—Effect of irradiation and packing treatments on the per cent weight loss of guavas is illustrated in Fig. 1. The irradiated and control fruits lost moisture almost to the

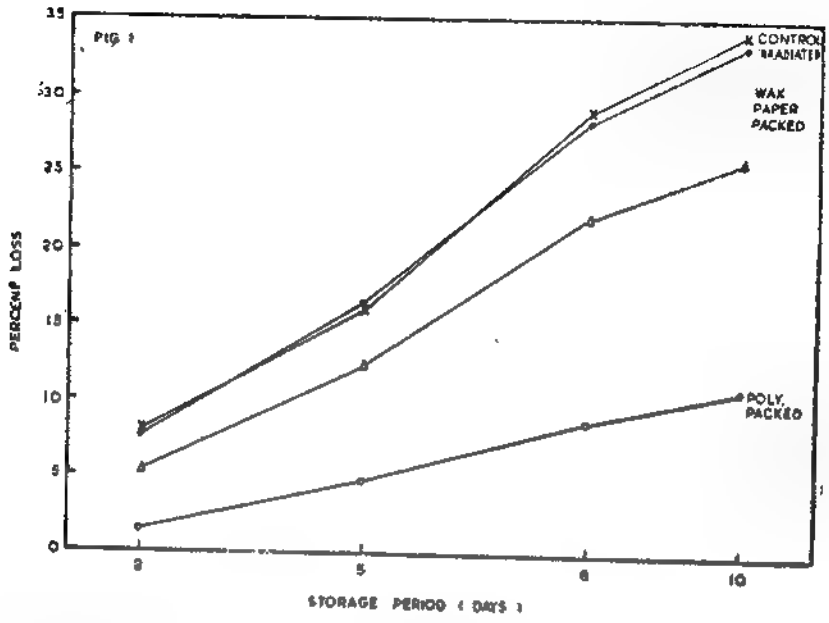

FIG. 1. Effect of gamma radiation (30 Krad), wax paper and polyethylene film packing on the weight loss of guavas during ripening at room temperature.

same level, but polyethylene film and wax paper packing caused a significant ($P<0.01$) reduction in weight loss. The weight loss of fruits on the 10th day was 34 per cent, 33 per cent, 26 per cent, and 11 per cent in the control, irradiated, wax paper-wrapped and polypacked guavas, respectively. The loss in weight was mainly due to evaporation and transpiration. Both types of packing retarded these processes and in this way loss in weight was decreased. Scott *et al* (1971) also showed that packing of bananas in polyethylene bags reduced weight loss during transport. The effect of irradiation on weight loss is controversial. Mathur and Lewis (1961) reported less weight loss in irradiated mangoes than in unirradiated mangoes but Herrera and Valencia (1964) found no effect of irradiation on the weight loss of this fruit.

Biochemical studies. –Effect of different doses of gamma radiation on the biochemical constituents of guavas during storage is discussed below.

a *Pectic substances.*—Radiation caused an immediate increase in water-soluble and ammonium oxalate-soluble pectins

and a corresponding decrease in alkali-soluble pectin (Table 1). This shows that there was an increase in soluble to insoluble

TABLE 1. *Effect of gamma radiation on different pectin fractions and total pectin (per cent AGA) of guavas during ripening at room temperature.*

Radiation dose (Krad)	H_2O soluble W	$NH_4C_2O_4$-soluble (A)	NaOH-soluble (N)	Total pectin	(W)[1] A+N
Immediately after irradiation					
0	0.150	0.075	0.815	1.040	0 169
10	0.145	0.060	0.765	0.970	0 176
20	0.170	0.075	0.770	0.995	0.178
30	0.175	0.090	0 750	1.015	0 208
40	0.170	0.100	0 740	1.010	0 202
50	0.205	0 090	0 715	0.010	0 255
75	0.220	0 110	0 690	0.020	0.275
After 5 days storage					
0	0.220	0.060	0.750	1 030	0.272
10	0.200	0.075	0.750	1 025	0.242
20	0.180	0.075	0.765	1.020	0 214
30	0.190	0.100	0.750	1.040	0 224
40	0.205	0.090	0.730	1.025	0.250
50	0.210	0.100	0 700	1.010	0.263
75	0.215	0.110	0.685	1 000	0.270
After 10 days storage					
0	0.350	0.110	0 380	0.840	0.714
10	0.345	0.100	0.411	0 856	0.675
20	0.330	0.110	0.412	0.852	0.632
30	0.305	0.110	0.450	0.865	0.545
40	0.310	0.100	0.415	0.855	0.569
50	0.325	0.130	0 412	0.867	0.600
75	0.345	0 150	0 412	0 907	0.614

[1] Soluble to insoluble pectin ratio.

pectin ratio in guavas with increasing dosage of irradiation. These changes prove the hypothesis proposed by Skinner and Kertesz in 1960 and Somogyi and Romani in 1964. Delayed ripening of guavas due to irradiation was reflected in diminished amounts of water-soluble pectin and a corresponding decrease in alkali-soluble pectin during the storage period Thus the normal conversion of insoluble to soluble pectins during ripening appears to have been markedly retarted by irradiation. Total pectin calculated by adding the different pectin fractions remained almost constant after irradiation but

there was a significant ($P<0.01$) decrease in this constituent after 10 days storage. Despite the softening which occurred due to a direct effect of irradiation, irradiated guavas retained firmer-than-normal texture at the end of the storage period. Similar effects of irradiation on the pectin constituents of fruits were obtained on apples (Massey *et al*, 1964); mangoes (Dennison and Ahmed, 1967); and peaches (Shewfelt *et al*, 1968).

b. *Ascorbic acid.*—It is clear from Table 2 that guavas contain appreciable quantities of ascorbic acid. There was a significant ($P<0.01$) decrease in vitamin C contents of guavas during ripening in storage at 25°C. Irradiation caused some destruction of ascorbic acid at higher radiation dose levels im-

TABLE 2.—*Effect of gamma radiation on the chemical constituents of guavas during ripening at room temperature.*

Radiation dose (Krad)	TSS Per cent	Acidity Per cent	TSS/acidity (ratio)	Ascorbic acid (mg/100 g)
Immediately after irradiation				
0	2.25	0.77	2.92	260
10	2.37	0.80	2.96	260
20	2.25	0.83	2.71	245
30	2.25	0.80	2.81	242
40	2.37	0.78	3.04	241
50	2.37	0.75	3.16	234
75	2.50	0.85	2.91	220
After 5 days storage				
0	3.00	0.64	4.60	185
10	3.25	0.64	5.08	183
20	3.00	0.65	4.62	186
30	3.27	0.60	5.45	182
40	3.25	0.58	5.68	183
50	3.25	0.61	5.33	181
75	3.37	0.62	5.44	175
After 10 days storage				
0	4.45	0.40	10.63	134
10	4.00	0.45	8.89	132
20	4.37	0.42	10.40	143
30	4.00	0.43	9.30	140
40	4.00	0.41	9.76	138
50	4.25	0.42	10.12	135
75	4.37	0.45	9.71	136

mediately after irradiation. At 75 Krad, the guavas contained 220 mg/100 g ascorbic acid while control fruit had 260 mg/100 g. This difference in control and irradiated fruits disappeared during subsequent storage period and the ascorbic acid contents were between 134 to 143 mg 100 g after 10 days storage.

c. *Total soluble solids (TSS) and acidity.*—There was significant ($P<0.01$) increase in TSS, TSS/ acid ratio and significant ($P<0.01$) decrease in acidity of guavas during ripening at room temperature (Table 2). Radiation treatment had no direct and delayed effect on these parameters. The increase in TSS of guavas is due to increase in sugars at the expense of starch which disappears during the ripening process. The decrease in acidity may be due to conversion of organic acid to other metabolites like esters etc. which are also responsible for the flavor development in the fruits. TSS/acid ratio increased during ripening due to an increase in TSS and a decrease in acidity, thereby resulting in sweeter fruits.

Microbiological studies.—Microbial spoilage of guavas was evident by the presence of fungi on the fruit imparting different colors to it depending on the type of fungal attack. These micro-organisms were isolated on malt extract agar (pH 5.0) and were identified. The decaying fruits were mainly attacked by *Aspergillus niger* and yeasts. The presence of this fungus blackened the surface of guavas. Other fungi were also present but to a lesser extent. These include *Cylindrocarpon* sp., *Papulaspora* sp., and *Trichothecium roseum*. The presence of the latter fungus gave pink color to the decaying guavas. Since the highest radiation dose used in the present investigation was 75 Krad, the spoilage fungi could not be controlled. According to Maxie and Sommer (1964) growth of fungi can only be reduced by dose levels of 175 Krad and above.

Organoleptic evaluation.—The effect of different doses of gamma radiation and packing treatments on the general acceptability of guavas is presented in Fig. 2. Organoleptic evaluation was carried out by 10 trained judge after 10 days storage. Mean scores for polyethylene film and wax paper-packed guavas were 7.75 and 7.00, respectively. From irradiated lots, 20, 30 and 40 Krad-treated fruits scored 5.88, 7.13 and 6.50, respectively. All other samples were disliked by the judges due to overripening, spotting and schrivelling of guavas

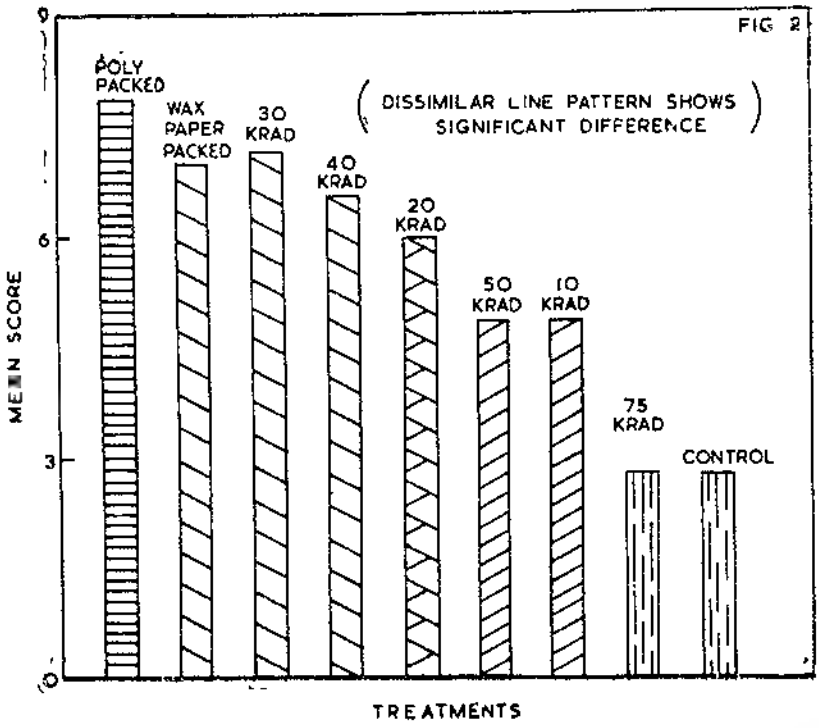

FIG. 2. Organoleptic evaluation of control, irradiated, wax paper and polyethylene film packed guavas after 10 days storage using hedonic scale scoring (average of 10 judges).

SUMMARY AND CONCLUSION

Gamma radiation (30 Krad), wax paper and polyethylene film packing extended the post-harvest life of guavas for 5, 6, and 7 days, respectively, over the control fruits. Organoleptic evaluation showed that after 10 days storage those guavas which were packed in polyethlene film were considered best. Packing treatments reduced the weight loss of guavas during storage. There was an increase in soluble to insoluble pectin ratio in guavas due to irradiation but it was reversed during subsequent storage. Other biochemical parameters studied were total soluble solids, acidity (and the ratio of the two) and ascorbic acid. Microorganisms isolated from decaying guavas were *A. niger*, *Cylindrocarpon* sp., *Papulaspora* sp., *Trichothecium roseum* and yeasts.

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REFERENCES

A. O. A. C. (1970) Official Methods of Analysis. P. O. Box 540, Benjamin, Franklin Station, Washington, DC 20044, 367 389.

Aziz, F., and P. J. Dyne (1963). A practical course in radiation chemistry, A. E. C. Lahore, 40 pp.

Chughtai, M. I. D., and A. W. Khan (1960). Nutritive value of foodstuff and planning of satisfactory diet in Pakistan. Part I. Composition of raw foodstuffs Division of Biochemistry, Institute of Chemistry, Punjab University, Lahore, Pakistan, 12 pp.

Cochran, W. G., and G. M. Cox (1965). Experimental Designs, 2nd Edition. New York. John Wiley and Sons., Inc, 95–105.

Dennison, R. A., and E. M. Ahmed (1967). Irradiation effect on the ripening of Kent mangoes. Jour. Food Sci. 32: 702–705.

Farooqi, W. A., M. Ali, and A. Muhammed (1967). Effect of gamma radiation on guavas (Psidium guajava L.). Food Irrad. 8: 37 40.

Glegg, R. E., F. P. Boyle, L. W. Tuttle, W. E. Wilson, and Z. I. Kestesy (1956). Effect of ionizing radiation on plant tissues. I. Quantitative measurement of softening of apples, beets and carrots. Rad Res. 5: 127 133

Herrera, J. R., and I. G. Valencia (1964) Preliminary report on the ripening of gamma irradiated mangoes Philippine Atomic Res. Centre, PAEC, Manila, Philippines.

Kertesy, Z. I., R. E. Glegg, G. F. Boyle, G. F. Parsons, and L. M. Massey, Jr. (1964). Effect of ionizing radiation on plant tissues. III. Softening and changes in pectin and cellulose of apples, carrots and beets. Jour. Food Sci. 29: 40-48.

Larmond, E. (1970). Methods for sensory evaluation of food. Food Research Institute, Central Experimental Farm, Ottawa, Canada. Dept. Agri. Publ. 1281: 36.

Mathur, P. B. (1963). Low dose gamma radiation of fresh fruits in India. Food Irrad. 4: A26–A28.

Mathur, P. B., and N. F. Lewis (1961). Storage behaviour of gamma-radiated mangoes Int. Jour. Appl. Radiat. Isotop. 11: 43 45.

Maxie, E. C., and N. F. Sommer (1964). Status of food irradiation as a technology for perishable commodities. Proc. Fruit Veg. Handl Conf., Univ. California, Davis, pp. 96 100

MAXIE, E. C., and N. F. SOMMER (1965). Irradiation of fruits and vegetables. In Radiation Preservation of Foods. Proc. Int. Conf. Boston, Massachusetts, Sept. 27–30 (1964) NAS/NRC 1273, Washington, D.C., pp. 39–52.

MASSEY, L. M., Jr., G. D. PARSONS, and R. M. SMOCK (1964). Radiation processing of foods. Some effects of gamma radiation on the keeping quality of apples. Agri. Food Chem. 12: 268–274.

MUNSELL, N. E., R. CASTILLO, C. ZURITA, and J. M. PORTILLS (1953). Production, uses and composition of food of plant origin from Ecuador. Food Res. 18: 319–314.

NAVIA, J. M., H. LOPEZ, M. CIMADEVILLA, E. FERNANDEZ, A. VALIENTE, I. D. CELEMENT, and R. B. HURRIS (1955). Nutrient composition of Cuban foods. I. Foods of vegetable origin. Food Res. 20: 97–113.

ROUSE, A. H., and C. D. ATKINS (1955). Pectinesterase and pectin in commercial citrus juice as determined by methods used at the Citrus Experiment Station. Univ. Florida Agri. Expt. Sta. Gainesville, Florida. Tech. Bull. 570: 1–19.

SATTAR, A., M. ALI, W. A. FAROOQI, and A. MUHAMMFI (1970). Radiation preservation of fruits and vegetables in Pakistan. Food Preservation Division, Radiation Genetics Institute, PAEC, Lyallpur, Pakistan, pp. 15–27.

SCOTT, K. J., and E. A. ROBERTS (1966). Polyethylene bags to delaying ripening in bananas during transport and storarge. Aust. Exp. Agric. Anim. Husb. 6: 197–199.

SCOTT, K. J., and E. A. ROBERTS (1968). New post-harvest treatments reduce wastage in bananas during marketing. Agri. Gaz. of N.S.W. 78: 605–607.

SCOTT, K. J., J. R. BLAKE, G. STRAGHAN, B. L. TUGWELL, and W. B. MC. GLASSON (1971). Transport of bananas at ambient temperature using polyethylene bags. Trop. Agric. (Trinidad, 48: 245–254.

SHEWFELT, A. L., E. M. AHMED and R. A. DENNISON (1968). Gamma radiation and storage treatments influence on pectic substances in peaches. Food Technol 22: 111–113.

SKINNER, E. R., and Z. I. KERTESZ (1960). The effect of gamma radiation on the structure of pectin; an electrophoretic study. Jour. Polymer Sci. 47: 99–109.

SMOCK, R. M., and A. H. SPARROW (1957). A study on the effect of gamma radiation on apples. Proc. Am. Soc. Hort. Sci. 20: 67–69.

SOMOGYI, L. P., and R. J. ROMANI (1964). Irradiation induces textural changes in fruits and its relation to pectin metabolism. Jour. Food Sci. 29: 266–371.

SURVIVAL OF RHIZOBIUM JAPONICUM IN INDIAN SOILS

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ABSTRACT

Period of survival of two strains of *Rhizobium japonicum*, U.S.D.A. 136 and Nanking, in seven soils of India from Delhi, Pusa, Dehra Dun, Ranchi, Akola, and Coimbatore (both red and black) was determined under different conditions. The determinations were made in sterilized constant moisture conditions and under undisturbed conditions after allowing the soils to dry gradually at room temperature. Similar determinations were also carried out in unsterilized soil under constant moisture and undisturbed conditions. It was observed that the counts gradually decreased with progress of time and that the counts and time were significantly correlated. The maximum period of survival of both strains was found under sterilized and constant moisture conditions and the minimum under unsterilized conditions when no moisture was maintained. The maximum survivability in soil was observed in the case of *Rhizobium japonicum*—Nanking variety in Ranchi soil which was 42 weeks and only 10 weeks in Coimbatore red soil. For both *Rhizobium japonicum*, U.S.D.A. 136 and Nanking varieties, the survivability was negatively correlated with pH but positively with available phosphorus (P) content of the soils. The correlations were highly significant.

INTRODUCTION

Survival of *Rhizobium* in soil under varying conditions is an important object of study from the point of view of cultivation of legumes and success of inoculation practices. The factors responsible for the protection of the organisms against adverse factors are not very well known. Some of the factors that influence the survival of *Rhizobium* are air, moisture, pH, temperature, soil type, soil composition, organic matter, micronutrient and antagonistic factors (Hedlin and Newton, 1948; Russel, 1952; Sen and Sen, 1956; Date, 1959; Bowen and Kennedy, 1959; Holland and Parker, 1962; Holding and King, 1963, Jardin and Samuel, 1963: Korovin and Vorobev, 1967).

In the present investigation, two strains of *Rhizobium japonicum* isolated soybean (*Glycine max.*), U.S.D.A. 136 and

080289——6

Nanking, were introduced in seven different soils with varying composition under sterilized and unsterilized and under controlled conditions. The periods of survival of the strains in the soils were determined. The results were examined to find out if the soil composition or the different conditions to which the soils were subjected, influenced the survival of the organism.

MATERIALS AND METHODS

Rhizobium culture.—Two efficient cultures of *Rhizobium japonicum* from soybean, strains-U.S.D.A. 136 and Nanking, were obtained from the culture collection of the Division of Microbiology, I.A.R.I., New Delhi.

Soil samples. The following seven soils were used as the carrier medium, Delhi (alluvial); Pusa (calcareous); Ranchi (red loam); Dehra Dun (hill); Akola (black cotton); and Coimbatore (red and black).

Preparation of inoculum and soil sample for culture.—Standard yeast-extract-mannitol medium was used for growing *Rhizobium* cultures. They were continuously shaken in a rotary shaker for 148 hours at 28 to 30 C. For each treatment 200 g of each soil sample were placed in four 500-ml Erlenmeyer flasks after drying and passing through 2-mm sieve. Moisture was made up to 33 per cent of their total water-holding capacities. This moisture level included the water carrying the suspension of nodule bacteria.

Out of four replications of each soil sample, two flasks were sterilized in the autoclave for 3 successive days at 120° C under 15 lbs for 2 hours each day. The flasks were inoculated aseptically with 20 ml of respective strain of *Rhizobium* (having an initial count 50 x 10^{10}/ml for U.S.D.A. 136 and 75 x 10^{10} ml for Nanking) and the soils mixed thoroughly. The prepared soil culture having one sterilized and one unsterilized flasks were kept at room temperature. Another pair of sterilized and unsterilized flasks were kept at 29°C. The flasks stored at 29°C were lightly stoppered and covered with cellophane paper to prevent too rapid loss of moisture. In the sterilized sample, the moisture was maintained throughout the storage period whereas in the unsterilized they were allowed to dry progressively at room temperature.

COUNTING METHODS

Survival counts of rhizobia in different soils were made forthnightly on the congo-red yeast-extract-mannitol agar (Fred *et al*, 1932; Hahn, 1966). Means were calculated from duplicate counts of four replications.

Plant dilution counts were made substantially according to the method of Date and Vincent (1962) by inoculating duplicate tubes of seedlings of *Phaseolus autropurpureus* (Siratro) as the test plant with 0.1 ml amounts of appropriate dilutions. The method was used in addition to the plate count when it was thought that the proportion of rhizobia to other organisms from unsterilized soil might be so low as to make counting difficult. Means were calculated from estimates of the same number of treatment replicates as were used in the plate count. Results from the two methods were in very good agreement and those quoted in tables below are based almost entirely on the plate count method. The period of survival in each soil was determined from the regression equations. The period at which the counts were zero, was taken to be the period of survival.

RESULTS

The soils were analyzed by the usual methods of soil analysis (Piper, 1950). The results of the observations are given in Table 1.

TABLE 1.—*Analysis of soil based on oven-dry samples.*

Place	Type of soil	Water-holding capacity	Soil reaction pH	Soluble salt (E. C.) mhos cm.	Organic C Per cent	CaCO Per cent	Available P K
Delhi	Sandy loam (alluvial riverine)	34 2	7 4	1.40	0 30	Nil	1 31
Pusa	Loam (alluvial riverine)	40 6	7.8	0.15	0.60	13 5	1 04
Dehra Dun	Loam	38 4	6 0	0 1.	0 89	Nil	13 0
Ranchi	Red loam	38 8	6.0	0.1.	0.40	Nil	
Akola	Clayey (Black)	37 2	7.7	0 20	0 71	4 8	
Coimbatore	Clay loam (red)	36 4	8 5	0 1.	0 48	Nil	
Coimbatore	Clayey (black)	57.2	8 1	1 40	0 .5	8 .	4 05

The counts of a strain of *Rhizobium japonicum* (U.S.D.A. 136) isolated from soybean in the different soils under sterilized and unsterilized conditions and under constant moisture

conditions and also in the natural state when no constant moisture was maintained at different periods are given in Table 2.

The results in Table 2 showed that there was progressive decrease in the counts with progress of time in all soils and also under different conditions. The correlation coefficients between the counts and the periods in weeks were all negative and highly significant. The periods of survival of the *Rhizobium* were different under some conditions and similar in others. In the majority of cases, the maximum period of survival was observed in sterilized soil under constant moisture conditions except in the soil from Ranchi where maximum period of survival was under sterilized condition but in undisturbed state. In the case of Pusa calcareous soil the period of survival was 12 weeks under varied conditions while in the case of black soil from Coimbatore it was 12 weeks in sterilized soil under constant moisture condition.

TABLE 2.—*Survival of Rhizobium japonicum (soybean U.S.D.A. 136) in different soils of India.*

Storage period in weeks	Number of rhizobia per g of soil (mean of four replications)			
	Sterilized soil		Natural soil	
	At 28°C sealed no drying	At room temperature unsealed progressive drying	At 28° C sealed no drying	At room temperature unsealed progressive drying
	Delhi soil pH 7.4			
2	97 x 10⁵	150 x 10⁵	72 x 10⁵	86 x 10⁵
4	35 x 10⁵	78 x 10⁵	40 x 10⁵	7 x 10⁵
6	10 x 10⁵	12 x 10⁵	8 x 10⁵	12 x 10⁵
8	36 x 10⁵	23 x 10⁵	—	—
10	13 x 10⁵	5 x 10⁵		
12	—	—		
r	−0.96	−0.92	−0.52	−0.82
Period of survival (weeks)	15	14	10	10
	Pusa soil pH 7.8			
2	169 x 10⁵	200 x 10⁵	99 x 10⁵	100 x 10⁵
4	41 x 10⁵	70 x 10⁵	23 x 10⁵	48 x 10⁵
6	13 x 10⁵	22 x 10⁵	7 x 10⁵	12 x 10⁵
8	41 x 10⁵	25 x 10⁵	15 x 10⁵	12 x 10⁵
10	—	—	—	—
12	—	—	—	—
r	−0.92	−0.93	0.90	0.91
Period of survival (weeks)	12	12	12	12

TABLE 2.—*Survival of rhizobium japonicum (soybean U.S.D.A. 136) in different soils of India* Continued.

Storage period in weeks	Number of rhizobia per g of soil (mean of four replications)			
	Sterilized soil		Natural soil	
	At 28° C sealed no drying	At room temperature unsealed progressive drying	At 28° C sealed no drying	At room temperature unsealed progressive drying
	Ranchi soil pH 6.0			
2	151 x 10^8	167 x 10^8	119 x 10^6	85 x 10^6
4	93 x 10^8	43 x 10^8	15 x 10^7	88 x 10^5
6	49 x 10^8	20 x 10^8	82 x 10^5	9 x 10^5
8	37 x 10^8	18 x 10^8	46 x 10^5	3 x 10^5
10	26 x 10^8	20 x 10^8	17 x 10^5	11 x 10^5
12	—	—	—	—
r	−0.80	−0.81	−0.87	−0.90
Period of survival (weeks)	16	17	15	14
	Debra Dun soil pH 6.0			
2	28 x 10^8	35 x 10^8	132 x 10^6	240 x 10^6
4	38 x 10^8	91 x 10^8	25 x 10^6	40 x 10^5
6	88 x 10^7	27 x 10^8	73 x 10^5	21 x 10^5
8	51 x 10^8	31 x 10^8	21 x 10^5	13 x 10^5
10	30 x 10^8	18 x 10^8	4 x 10^5	3 x 10^5
12	42 x 10^8	35 x 10^8	4 x 10^5	—
r	−0.93	−0.91	−0.77	−0.86
Period of survival (weeks)	36	31	31	15
	Akola soil ph 7.7			
2	155 x 10^8	60 x 10^8	61 x 10^6	32 x 10^6
4	74 x 10^8	38 x 10^8	245 x 10^5	241 x 10^5
6	12 x 10^8	12 x 10^8	20 x 10^5	16 x 10^5
8	44 x 10^8	33 x 10^8	83 x 10^5	—
10	—	—	—	—
12	—	—	—	—
r	−0.85	−0.91	0.89	−0.91
Period of survival (weeks)	12	12	12	11
	Coimbatore red soil pH 8.5			
2	123 x 10^8	142 x 10^8	76 x 10^5	3 x 10^5
4	10 x 10^8	25 x 10^8	30 x 10^5	11 x 10^4
6	50 x 10^8	79 x 10^8	79 x 10^5	7 x 10^4
8	22 x 10^8	39 x 10^8	—	41 x 10^4
10	—	—	—	—
12	—	—	—	—
r	0.90	0.89	−0.89	0.82
Period of survival (weeks)	12	11	10	8

NOTE: The blank spaces in the table mean absence of any colony at the time of counting.

TABLE 2.—*Survival of rhizobium japonicum* (*soybean U.S.D.A. 136*) *in different soils of India*—Continued.

Storage period in weeks	Number of rhizobia per g of soil (mean of four replications)			
	Sterilized soil		Natural soil	
	At 25°C sealed no drying	At room temperature unsealed progressive drying	At 25°C sealed no drying	At room temperature unsealed progressive drying
	Combatore red soil			
2	188 x 10^6	262 x 10^6	77 x 10^6	47 x 10^6
4	23 x 10^6	35 x 10^6	25 x 10^6	28 x 10^6
6	8 x 10^6	11 x 10^6	16 x 10^6	14 x 10^6
8	69 x 10^6	28 x 10^6	28 x 10^6	14 x 10^6
10	—	—	—	—
12	—	—	—	—
Period of survival (weeks)	—0.89 12	—0.94 11	—0.94 11	—0.94 11

NOTE: The blank spaces in the table mean absence of any colony at the time of counting.

Counts of Nanking strain of *Rhizobium japonicum* under sterilized and unsterilized conditions in the presence and absence of constant moisture contents at different periods are given in Table 3.

The results for Nanking strain in Table 3 indicated the same trend as in strain U.S.D.A. 136. However, in the case of Ranchi soil, the highest period of survival was 42 weeks as compared to 17 weeks in strain U.S.D.A. 136. In the red soil from Coimbatore, the period of survival was the same in all cases.

From the results of the present studies it can be seen that even under most suitable conditions, the maximum period of survival in the case of *Rhizobium* for soybean varied from 36 to 42 weeks and the minimum period of survival from 8 to 10 weeks. The maximum period of survival was found under sterile and constant moisture conditions and the minimum under unsterilized conditions when no moisture was maintained. This is in accordance with the observations of Hedlin and Newton (1948) who have shown that the counts of bacteria in soil is closely related to the moisture content. Also under sterilized conditions the competition with the other micro-organisms or antagonism between the introduced *Rhizobium* and the indigenous microflora were absent. However, survival of the organism from soybean has been reported by several workers

TABLE 3.—*Survival of Rhizobium japonicum (Nanking) in different soils in India.*

Storage period in weeks	Number of rhizobia per g of soil (mean of four determinations): Sterilized soil — At 29°C sealed no drying	Sterilized soil — At room temperature unsealed progressive drying	Natural soil — At 29°C sealed no drying	Natural soil — At room temperature unsealed progressive drying
	Delhi soil pH 7.4			
2	28×10^{5}	$26 \times 10^{[illegible]}$	$22 \times 10^{[illegible]}$	22×10^{5}
4	$11 \times 10^{[illegible]}$	112×10^{5}	37 x 10	[illegible] x $10^{[illegible]}$
6	30 x 10	111×10^{6}	22 x 10	$22 \times 10^{[illegible]}$
8	10 x 10	—	—	
10	—	—	—	
12	—	—	—	—
r	0.31	−0.88	−0.90	0.92
Period of survival (weeks)	12	10	10	15
	Pusa soil pH 7.8			
2	$25 \times 10^{[illegible]}$	30×10^{6}	15 x 10	61×10^{5}
4	$9[illegible] \times 10^{6}$	37×10^{5}	30×10^{5}	$2[illegible] \times 10^{5}$
6	33×10^{6}	13×10^{5}	93 x 10	2[illegible] x 10
8	36 x 10	11×10^{5}	[illegible]7 x 10	4 x 10
10	29 x 10	12 x 10	—	
12	—		—	—
r	−0.86	−0.8[illegible]	−0.89	0.9[illegible]
Period of survival (weeks)	15	14	12	10
	Ranchi soil pH 6.0			
2	$12[illegible] \times 10^{6}$	$1[illegible]3 \times 10^{[illegible]}$	$20[illegible] \times 10^{[illegible]}$	113×10^{5}
4	$1[illegible]9 \times 10^{6}$	127×10^{6}	48×10^{5}	99×10^{5}
6	$1[illegible] \times 10^{[illegible]}$	19 x 10	$21 \times 10^{[illegible]}$	$3[illegible] \times 10^{5}$
8	144×10^{5}	$92 \times 10^{[illegible]}$	[illegible]1 x 10	$[illegible] \times 10^{5}$
10	$20[illegible] \times 10^{5}$	64 x 10	97 x 10	$30 \times 10^{[illegible]}$
12	$11[illegible] \times 10^{[illegible]}$	32 x 10	$98 \times 10^{[illegible]}$	$1[illegible] \times 10^{[illegible]}$
r	−0.[illegible]6	0.94	0.7[illegible]	0.7[illegible]
Period of survival (weeks)	42	41	40	34
	Dehra Dun soil pH 6.0			
2	$33 \times 10^{[illegible]}$	$[illegible]0 \times 10^{[illegible]}$	[illegible] x 10	21×10^{6}
4	$1[illegible]0 \times 10^{[illegible]}$	$[illegible]6 \times 10^{[illegible]}$	11[illegible] x 10	$1[illegible]5 \times 10^{[illegible]}$
6	[illegible] x 10	1[illegible] x 10	[illegible]	[illegible]
8	$31 \times 10^{[illegible]}$	17 x 10	$4[illegible] \times 10^{[illegible]}$	$[illegible]3 \times 10^{[illegible]}$
10	117 x 10	[illegible] x 10	36 x 10	$18 \times 10^{[illegible]}$
12	$[illegible] \times 10^{[illegible]}$	[illegible] x 10		
r	−0.[illegible]8	[illegible].8[illegible]	[illegible]	0.86
Period of survival (weeks)	2[illegible]	31	16	15

TABLE 3 *Survival of Rhizobium japonicum (Nanking) in different soils in India*—continued.

Storage period (weeks)	Number of rhizobia per g of soil, mean of four replications			
	Sterilized soil		Natural soil	
	At 28°C sealed to dry ng	At room temperature unsealed progressive drying	At 28°C sealed drying	At room temperature unsealed progressive drying
	Akola soil pH 7.7			
2	6[illegible] x 10[illegible]	23 x 10[illegible]	46 x 10[illegible]	36 x 10[illegible]
4	137 x 10[illegible]	55 x 10[illegible]	89 x 10[illegible]	1 x 10[illegible]
6	57 x 1[illegible]	37 x 10[illegible]	30 x 10	15 x 10
8	29 x 10[illegible]	7 x 10[illegible]	—	—
10	—	—	—	—
12	—	—	—	—
Period of survival (weeks)	—0 88 12	0 89 12	—0 91 12	0 91 11
	Coimbatore red soil pH 8.5			
2	6[illegible] x 10[illegible]	34 x 10[illegible]	28 x 10[illegible]	4[illegible] x 10[illegible]
4	7[illegible] x 10[illegible]	79 x 10[illegible]	17 x 10[illegible]	13 x 1[illegible]
6	6 x 10[illegible]	2 x 10[illegible]	38 x 10[illegible]	24 x 10[illegible]
8	—	—	—	—
10	—	—	—	—
12	—	—	—	—
Period of survival (weeks)	0 92 10	0 93 10	0 87 10	—0 9 10
	Coimbatore black soil pH 8.1			
2	153 x 10[illegible]	109 x 10[illegible]	47 x 10[illegible]	64 x 1[illegible]
4	77 x 10[illegible]	51 x 10[illegible]	18 x 10[illegible]	12 x 1[illegible]
6	1[illegible] x 10[illegible]	[illegible] x 10[illegible]	2[illegible] x 10[illegible]	4 x 1[illegible]
8	5[illegible] x 10	78 x 10	—	—
10	—	—	—	—
12	—	—	—	—
Period of survival (weeks)	0 92 12	0 92 12	—0 91 10	0 9 1[illegible]

to be quite prolonged (Richmond, 1926; Vandecaveye, 1927; Thornton, 1943; Newbould, 1951; Sen and Sen, 1956; Thompson, 1964; Choudhury, 1965). The variations in the survivability of different strains of *Rhizobium* in different soils under different conditions show that a study of the behavior of an individual strain is imperative not only for using a particular soil as a carrier for a particular strain as a legume inoculant but also for finding out if the inoculation is going to succeed in the case of a particular crop in a particular soil.

The values for the two *Rhizobium* strains on Ranchi soil (pH 6 0) are widely divergent, one being about 16 weeks and the other 40 weeks. This shows the superiority of the survival of one strain over the other compared with other soils having pH values above 7.0 where survivability are fairly closely grouped.

In the case of *Rhizobium japonicum,* U.S.D.A. 136 and Nanking varieties, correlation could be obtained between soil pH and available P with survivability. In the case of U.S.D.A. 136 the relationship between soil pH and survivability ($r = -0.80$) and that between available P and survivability ($r = +0.76$) were found to be significant. The relationships can be expressed as

$$Y = 82.32 - 8.02\ X \text{ and}$$
$$Y = 8.22 + 0.69\ X_1$$

where Y is the longevity in weeks in both cases while X and x' are the pH and available p content of the soils, respectively. Similarly in the case of Nanking variety, the relationship between soil pH and survivability ($r = -0.82$) and that between available P and survivability ($r = +0.79$) were highly significant. The relationships can be expressed as

$$Y = 87.20 - 7.92\ X \text{ and}$$
$$Y = 7.90 + 0.68\ X_1$$

where Y is the longevity in weeks in both cases while X and and X_1 are the pH and available P content of the soils, respectively.

The results of the present study show that the low average survivability of strains of *Rhizobium* in soils and its variation according to the nature of the strain and soil would make determination of survivability of *Rhizobium* strains in a soil imperative when the soil is used as a carrier in the preparation of legume inoculants as is the practice in India. Also it is seen that an average strain of a species of *Rhizobium* in an Indian soil as carrier not likely to maintain sufficient counts by 12 weeks so as to be effective and beneficial. The low survivability of strain of *Rhizobium* in an average Indian soil may cause doubts about their existence on natural soils for long periods where the indigenous soil microflora may cause additional difficulties. A natural soil unlike one in the laboratory is highly dynamic and undergoes change

almost all the time. When a crop is grown the rhizosphere effect is prominent along with the conservation of moisture around the roots and exudates which may consist of stimulatory substances and a good deal of food materials for microorganisms. This may be quite prominent under native mixed vegetation. Even in bare soil, where there is no vegetation, there is rise of subsoil water bringing in and leaching out many organic and inorganic substances. There are, therefore, greater chances of survival of *Rhizobium* under natural conditions in the field rather than in the laboratory under artificial conditions.

ACKNOWLEDGMENT

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REFERENCES

BOWEN, G. D., and M. KENNEDY (1959). Effect of high soil temperature on Rhizobium spp. Qd. Jour. Agric. Sci. 16: 177–197.

CHOWDHURY, M. S. (1960). Growth and persistence of Rhizobium in soil. Ph.D. Thesis, Univ. West. Aust.

DATE, R. A. (1959). Growth and survival of the root nodule bacteria. M. Sc. Thesis, Univ. of Sydney.

DATE, R. A., and J. M. VINCENT (1962). Determination of the number of root-nodule bacteria in the presence of other organisms. Aust. Jour. Expt. Agri. Anim. Husb. 2: 5–7.

FRED, E. B., I. L. BALDWIN, and E. McCOY (1932). Root nodule bacteria and leguminous plants. Univ. Wisconsin. Studies in Science 1405: 343.

HAHN, N. J. (1966). The congo red reaction in bacteria and its usefulness in the identification of bacteria. Can. Jour. Microb. 12: 725–733.

HEDLIN, R. A., and S. D. NEWTON (1948). Some factors influencing the growth and survival of Rhizobium in humus and soil cultures. Canad. Jour. Research. (C) 26: 174–187.

HOLLAND, A. A., and C. A. PARKER (1962). Microbial intervention in the establishment of clover. VIII Intern. Cong. Microbial. (Abs.) pp. 22–23.

HOLDING, A. J., and J. KING (1963). The effectiveness of indigenous populations of Rhizobium trifolii in relation to soil factors. Pl. Soil 18: 191–198

JARDIM FREIRE, J. R., and H. J. SAMUEL 1963). Influence of temperature and loss of moisture on the death rate of Rhizobium inoculants. Agron. Vet. 6: 85.

KOROVIN, A. I., and V. A. VOROBEV (1967). Soil temperature and utilization of atmospheric nitrogen by legumes. Fiziol. Rast. 14: 117–122.

NEWBOULD, F. H. S. (1951). Studies on humus type legume inoculants I. Growth and survival in storage. Sci. Agri. 31: 463–469.

PIPER, C. S. (1950). Soil and plant analysis. New York: Intern. Sci. Pub. Inc., pp. 143–150.

RICHMOND, T. A. (1926). Longevity of the legume nodule organism. Jour. Amer. Soc. Agron. 18: 414–416.

RUSSELL, M. B. (1952). Soil aeration and plant growth. In B. T. Shaw (Ed.). Soil Physical Condition and Plant Growth. Chap. 4, p. 253.

SEN, A., and A. N. SEN (1956). Survival of Rhizobium japonicum in stores air-dry soil. Jour. Indian Soc. Soil Sci. 4: 215–220.

THOMPSON, J. A. (1964). Studies on the survival of root-nodule bacteria on seed and in soil. Ph.D. Thesis, Univ. of Sydney.

THORNTON, GEO, D. (1943). Some factors affecting the longevity of Rhizobium in Florida soils. Proc. Soil Sci. Soc. Amer. 8: 238–240.

VANDECAVEYE, S. C. (1927). Effect of moisture, temperature and other climatic conditions on Rhizobium leguminosarum in the soil. Soil Sci. 23: 355–362.

SHORT COMMUNICATION

THE OZONOLYSIS OF PHILIPPINE UNSATURATED OILS. II. LUMBANG [ALEURITES MOLUCCANA (LINN.) WILLD.]

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The previous publication in this series (Arida *et al*, 1967) described a two-step process for the production of a good technical grade azelaic acid from rice bran oil. We now wish to report the extension of the process to lumbang oil.

The conditions are essentially the same as for rice bran oil. Both the yield and the quality of the azelaic acid obtained from lumbang oil were better than that obtained from rice bran oil. Moreover, the nitric acid could be recycled indefinitely.

MATERIALS AND METHOD

Reagents.—These were as previously described. The lumbang oil obtained from Elizalde Oil and Paint Factory, Tanduay, Manila, was light yellow in color and used without purification.

Ozonolysis of lumbang oil. -A study was made on the effects of the various reaction parameters on the yield and purity of the product.

In a typical run, 20 g of lumbang oil was dissolved in a solvent consisting of 350 ml of hexane and 100 ml of ethanol and ozonized as previously described. The resulting mixture was refluxed with 400 ml of 3 per cent nitric acid for 1 hour. The solvent was distilled off and the resulting two-phase system separated into an oily supernatant layer and an aqueous layer. The former contained pelargonic and other acids and was not considered further.

The aqueous layer was cooled and the crystalline azelaic acid recovered by filtration. The material was crystalline white in

color with a melting point ranging from 92° to 106°C though the range for a given sample never exceeded 3 degrees. The melting point was raised from 99° to 100°C to 100° to 101°C upon one recrystallization from boiling water containing a few drops of ethanol with 92.5-per cent recovery.

The recovered solvent was reused. The dilute nitric acid from the filtration was brought up to 3 per cent with concentrated acid as previously described and reused in subsequent run. Unlike in the case of rice bran oil, we found no limit to the number of times this acid could be recycled.

RESULTS AND DISCUSSION

The lumbang oil is first ozonized in a hexane-ethanol medium to give what is probably an ethoxy hydroperoxide rather than an ozonide (Bailey, 1956). In the second step this is refluxed with dilute nitric acid, which serves both to oxidize the intermediate to the acid function and to hydrolyze the ester linkage.

Table 1 shows the results of a series of runs. The yield of the first run was low because of the solubility of azelaic acid in the water layer The average yield is over 25 per cent. This is considerably better than that obtained from rice bran oil and the quality of the product was better.

Attempts to improve the process by using less alcohol and by making various changes in the secondary step were not successful.

Since the starting material is a glycerol ester it would be expected that glycerol would build up in the water layer and interfere eventually with the process. Such an effect was not noticed in the 17 consecutive runs made. Moreover, in our hands, the application of this process to pure oleic acid did not give a higher yield of product when correction is made for the parts of lumbang oil (glycerol, acids other than oleic, linoleic and linolenic) which could not give azelaic acid on ozonolysis. Therefore, no economic benefit would be derived from prior hydrolysis of lumbang oil with subsequent ozonolysis of the isolated unsaturated fatty acids.

Contrary to the report of Magiollo (1958), we found that the presence of water in our solvent system lowered considerably the yield of final product obtained from the ozonolysis of oleic acid.

TABLE 1.—*Ozonolysis of lumbang oil.*

Run No.[1]	Yield of azelaic acid[2] *Per cent*
1	12.1
2	23.4
3	22.9
4	20.2
5	10.7
6	20.0
7	27.2
8	21.8
9	26.5
10	33.9
11	34.3
12	21.0
13	36.1
14	30.9
15	28.8
16	28.0
17	24.8
Average	25.4

1 Hexane and nitric acid recycled.
2 Based on commercial oil.

SUMMARY

The study on the ozonolysis of Philippine unsaturated oils has been extended to lumbang [*Aleurites moluccana* (Linn.) Willd.] oil. The azelaic acid obtained was white crystalline flakes, the quality being much better than that obtained from rice bran oil. The average yield was over 25 per cent.

REFERENCES

ARIDA, V.P. F.C. BORLAZA and W.J. SCHMITT, S. J. (1967). The ozonolysis of Philippine unsaturated oils. I. Rice-bran oil. Philip. Jour. Sci. 96: 249–252.

BAILEY, P.S. (1956). The ozonolysis of phenanthrene in methanol. Jour. Am. Chem. Soc. 78: 3811–3816.

MAGIOLLO, A. (1958). Dibasic and monobasic acids. U.S. Patent No. 2,865,937, Dec. 23. Cited in Chem. Abst. (1959) 53: 19992.

BOOK REVIEW

Books reviewed in this section represent a selection from those received occasionally by the National Institute of Science and Technology and the Philippine Atomic Energy Commission, two sister agencies under the National Science Development Board.

Scientific Thought. Some underlying concepts, methods and procedures. 1972. 24 x 15.5 cm, 252 pp. Price, $15 £5 60 F.

This book is a collection of articles by eminent scientists invited by Unesco to explain ideas, methods or procedures which underline extraordinary discoveries and developments of the twentieth century. *Scientific thought* attempts an interdisciplinary approach to problems common to both the natural sciences and the humanities, providing a philosophical framework in which these problems may be viewed.

A quotation from Professor Salam neatly crystalizes this link between philosophy and science:

> We have always found that whenever a postulated symmetry principle was appearing to fail in natural phenomena, this must be due to some still deeper symmetry, with which it must be in conflict. We may, at a given time, fail to comprehend the aesthetics of nature. When, however, the full and final picture emerges one has invariably found that the symmetries this exhibits are profounder still.

This search for the ultimate beyond the ultimate belongs to no one discipline; it is basic to many of the subjects treated in this book and engages both the philosopher and the scientist.

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PUBLICATIONS AVAILABLE

CHECKLIST OF THE ANTS (HYMENOPTERA: FORMICIDAE) OF ASIA. By J. W. Chapman and S.R. Capco. Institute of Science and Technology Monograph 1 (1951) new series. Paper, 372 pages. Price, $2.00, United States currency.

NOTES ON PHILIPPINE MOSQUITOES, XVI. GENUS TRIPTEROIDES. By F. E. Baisas and Adela Ubaldo-Pagayon. Institute of Science and Technology Monograph 2 (1952) new series. Paper, 198 pages with 23 plates and four text figures. Price $2.50, United States currency.

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